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(54) Title: IMMUNOLOGICAL REAGENT SPECIFICALLY INTERACTING WITH THE EXTRACELLULAR DOMAIN OF THE HUMAN ZETA CHAIN

(57) Abstract

The present invention relates to a nucleic acid molecule comprising a nucleic acid sequence encoding at least one complementary determining region (CDR) of a variable region of an antibody, said antibody specifically interacting with the extracellular domain of the human zeta–chain, said antibody being obtainable by immunizing a rat with Jurkat cells and subsequently with a conjugate comprising a carrier molecule and a peptide comprising the 11N–terminal amino acids of the rat zeta–chain. Preferably, the (poly)peptide encoded by the nucleic acid molecule of the invention is a monospecific or bispecific antibody. The invention also relates to pharmaceutical compositions comprising i.a. the nucleic acid molecule or antibody of the invention as well as to kits comprising the aforementioned compounds. Finally, the invention relates to a method for the determination of zeta–chain or eta–chain expression on NK–cells, T–cells or precursors thereof employing the antibody of the invention.

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Immunological reagent specifically interacting with the extracellular domain of the human zeta chain

The present invention relates to a nucleic acid molecule comprising a nucleic acid sequence encoding at least one complementary determining region (CDR) of a variable region of an antibody, said antibody specifically interacting with the extracellular domain of the human zeta-chain on the surface of intact cells, said antibody being obtainable by immunizing a rat with Jurkat cells and subsequently with a conjugate comprising a carrier molecule and a peptide comprising the 11 N-terminal amino acids of the rat zeta-chain. Preferably, the (poly)peptide encoded by the nucleic acid molecule of the invention is a monospecific or bispecific antibody. The invention also relates to pharmaceutical compositions comprising i. a. the nucleic acid molecule or antibody of the invention as well as to kits comprising the aforementioned compounds. Finally, the invention relates to a method for the determination of zeta-chain or eta-chain expression on NK-cells, T-cells or precursors thereof employing the antibody of the invention.

The zeta-chain is part of a family of structurally and functionally related signal transduction molecules, further encompassing the eta-chain (an alternatively spliced form of the zeta-chain) and the gamma-chain of the high affinity IgE-Fc-receptor FcεRI. Common traits within this family of transmembrane proteins are their long intracellular domain comprising one or several ITAM sequence motifs (immunoreceptor tyrosine-based activation motif; zeta: 3, eta: 2, gamma: 1) as well as extremely short extracellular domains, constituted of 9 (zeta, eta, sequence identical) or 4 (FcεRI-gamma) amino acids. The sequence of the extracellular domains of these proteins is 100% conserved between mouse, rat and man, and most likely other species as well.

The zeta-chain is expressed as a homodimer or as a heterodimer with the eta-chain on T-lymphocytes, natural killer- (NK-) cells and, to some extent, their precursors, exclusively. On the surface of mature T-lymphocytes the zeta chain is structurally and

functionally closely associated with the T-cell receptor (TCR) and the CD3-complex. Signals induced through engagement of the TCR are transduced into the cytoplasm of the T-cell via CD3 and the zeta-chain, with the three ITAMs of the zeta-chain providing the major part of the signal amplification effect compared to the single ITAM on the epsilon-, delta- and gamma-chain (different from FcεRI-gamma) that constitute the CD3-complex.

On the surface of NK-cells, the zeta-chain shows a similar association with the IgG-Fc-receptor (FcγRIIIA). When antibody-coated target cells are recognized by NK-cells via FcγRIIIA, the resulting signal is transduced to the cytoplasm through the zeta- and/or the gamma-chain, thus activating the NK-cell which consecutively lyses the target cell that was recognized (ADCC, antibody dependent cellular cytotoxicity).

The TCR-complex on mature T-lymphocytes is thus an oligomeric structure, composed of multiple chains (TCR-α/β or TCR-γ/δ associated with CD3ε, CD3δ, CD3γ and the zeta chain or its alternative splice-product eta) (Keegan, Immunology Today 13 (1992) 63-68). Antigen recognition is accomplished by the polymorphic TCR-α/β- or TCR-γ/δ-heterodimers that are devoid of intracytoplasmatic signal transduction domains. The invariant CD3 proteins (γ/ε- and δ/ε-heterodimers) and the zeta or eta chain (zeta-homodimers or zeta-eta-heterodimers) are necessary for correct assembly, transport and efficient cell surface expression of the whole TCR-complex and transduce TCR signals (Clevers, Annu. Rev. Immunol. 6 (1988) 629-662, Ashwell, Annu. Rev. Immunol. 8 (1990) 139-167). Signaling requires a conserved 18-amino acid sequence (Reth, Nature 338 (1989) 383-384, Samelson, J. Biol. Chem. 267 (1992) 24913-24916), termed the immunoreceptor tyrosine-based activation motif (ITAM), which is found three times in the zeta chain, twice in the eta chain and once in each of the CD3 subunits (γ, δ and ε). Each ITAM contains a pair of tyrosine-X-X-leucine/isoleucine (Y-X-X-L/I) motifs, that are separated by 10 or 11 amino acids (Cambier, Immunology Today 16 (1995) 110). The tyrosine residues in each ITAM are rapidly phosphorylated after TCR ligation and serve as docking sites for signaling proteins that can bind to the phosphotyrosine residues by src-homology-2 (SH2) domains (Cooke, Cell 65 (1991) 281-291, Glaischenhaus, Cell 64 (1991) 511-520, Samelson, Proc. Natl. Acad. Sci. USA. 87 (1990) 4358-4362, Straus, Cell

70 (1992) 585-593, Songyang, Cell 72 (1993) 767-778, Songyang, Mol. Cell. Biol. 14 (1994) 2777-2785, Isakov, J. Exp. Med. 181 (1995) 375-380). The necessity of the zeta-chain in TCR-mediated signaling was demonstrated by studies of a zeta-deficient mutant derivative of an antigen-specific T cell hybridoma; the mutant line was incapable of responding to antigen and only poorly responsive to anti-CD3 antibodies (Sussman, Cell 52 (1988) 85-95). Although the retention of some activity in response to anti-CD3 antibody stimulation suggested the other chains of the TCR-complex were able to compensate for the absence of zeta, studies in which the mutant line was shown to reacquire the ability to recognize antigen or to respond to anti-CD3 antibodies when reconstituted with zeta-chain by transfection clearly document an important role for the zeta-chain in signal transduction (Weissman, EMBO J. 8 (1989) 3651-3656). Due to its singular configuration, the zeta chain may function as the predominant TCR signaling structure and its triplicated ITAMs may serve primarily to facilitate TCR signal amplification.

The TCR-complex in general and the zeta-chain mediated signal transduction in particular are involved in both the activation and the programmed cell death (apoptosis) of mature T-lymphocytes. Experiments in which the zeta-chain cytoplasmatic tail was attached to unrelated receptors showed that cell lines expressing these chimeric receptors could respond to antibody crosslinking with IL-2 release and upregulation of other activation parameters (Irving, Cell 64 (1991) 891-901). Furthermore, cytotoxic T lymphocytes (CTL) expressing chimeric zeta-chain derivatives were shown to specifically lyse target cells bearing surface molecules recognized by the chimeric zeta-receptor (Romeo, Cell 64 (1991) 1037-1046, Romeo, Cell 68 (1992) 889-897). This, however, proved to be true only in case of activated T cells since resting T lymphocytes expressing chimeric zeta-chain molecules could neither be activated nor show any cytotoxicity against target cells when stimulated through these chimeric receptors (Brocker, J. Exp. Med. 181 (1995) 1653-1659), that according to biochemical analyses do not associate with endogenous TCR-subunits and therefore act as physically independent signaling molecules (Shinkai, Immunity 2 (1995) 401-411). These data thus indicate that chimeric zeta-chain derivatives can only substitute for the complete TCR-complex in activated but not in resting T cells.

TCR-mediated apoptosis of mature T lymphocytes may be induced if strong TCR reengagement occurs when the cells are activated and proliferating (Lenardo, Nature

353 (1991) 858-861, Russell, Proc. Natl. Acad. Sci. USA. 88 (1991) 2151-2157, Critchfield, Cell. Immunol. 160 (1995) 71-78). Mature T cell death plays a critical role in peripheral immune homeostasis and tolerance. Recent experiments show that the zeta-chain is required for efficient induction of T-cell apoptosis through engagement of the TCR and that the signaling domains of CD3 only have a minor (CD3ε) or no (CD3γ and δ) effect on TCR-mediated apoptosis (Combadière, J. Exp. Med. 183 (1996) 2109-2117). In addition, the three zeta-chain ITAMs contribute differently to the induction of T-cell apoptosis, with the most N-terminal one having the predominant effect followed by the C-terminal ITAM resembling the low contribution of CD3ε and the middle one being completely incapable of inducing apoptosis.

T cell development takes place primarily in the thymus, where T cell precursors immigrate from the fetal liver or from adult bone marrow. Upon immigration into the thymus these early progenitor T cells are triple negative (TN: TCR^- $CD4^-$ $CD8^-$) (Shortman, Annu. Rev. Immunol. 14 (1996) 29-47) but already express the zeta chain and CD3 chains (Wiest, J. Exp. Med. 180 (1994) 1375-1382, Wilson, Int. Immunol. 7 (1995) 1659-1664). In the inductive environment of the thymus they transit a series of developmental stages prior to their differentiation into $CD4^+CD8^+$ double positive (DP) thymocytes (Godfrey, Immunol. Today 14 (1993) 547-553). The most immature $CD44^+CD25^-$-TN-thymocytes and $CD44^+CD25^+$-TN-thymocytes derived therefrom still show germline configuration of the TCR-genes. TN-thymocytes of the next maturation stage characterized by the surface phenotype $CD44^{-/lo}CD25^+$, however, start to rearrange the TCRβ locus. Up to this stage the zeta chain, although expressed, is probably not required for thymocyte maturation (Crompton, Eur. J. Immunol. 24 (1994) 1903-1907). The following maturation step of TN-thymocytes is characterized by the phenotype switch from $CD44^{-/lo}CD25^+$ to $CD44^-CD25^-$, however, it is blocked in the absence of the zeta-chain (Crompton, Eur. J. Immunol. 24 (1994) 1903-1907) and requires rearrangement and expression of the TCRβ chain (Kishi, EMBO J. 10 (1991) 93-100, Mombaerts, Nature 360 (1992) 225-231, Shinkai, Science 259 (1993) 822-825). The TCRβ chain associates with an invariant chain termed pre-Tα (pTα) (Groettrup, Cell 75 (1993) 283-294), that substitutes for the still unrearranged TCRα chain to form the pre-TCR probably associated with the zeta-chain and CD3-chains

(Van Oers, J. Exp. Med. 182 (1995) 1585-1590). As a consequence of signals mediated by the pre-TCR, thymocytes progress in development to the DP stage. At this stage, thymocytes initiate rearrangement of their TCRα genes, cease to express pTα, and start to express low levels of TCR complexes, resembling the multiple chain composition of the TCR-complex on mature T-lymphocytes (Von Boehmer, Ann. N. Y. Acad. Sci. 766 (1995) 52-61, Robey, Annu. Rev. Immunol. 12 (1994) 265-705).

Since the development of $CD44^-CD25^-$-TN thymocytes and subsequently that of DP thymocytes is inhibited in the absence of the zeta-chain and can be restored by the expression of a signaling deficient mutant zeta-chain without functional ITAMs, respectively (Shores, J Immunol 159 (1997) 222-230, Shores, Science 266 (1994) 1047-1050), the importance of the zeta-chain related to the promotion of the pre-TCR surface expression may exceed that related to its signaling potential.

DP thymocytes are further subjected to selection on the basis of the specificity of their TCRs. Thymocytes expressing TCRs with negligible specificity for self-MHC molecules, irrespective of which peptide is bound by the MHC-protein, die within the thymus, presumably because they fail to receive survival signals through their TCR (Robey, Annu. Rev. Immunol. 12 (1994) 265-705, Jameson, Annu. Rev. Immunol. 13 (1995) 93-126). In contrast, thymocytes that express TCRs with the appropriate ligand specificities survive and mature to either $CD4^+$- or $CD8^+$-single positive (SP) T-cells showing high level TCR-expression. However, thymocytes expressing autoreactive or potentially autoreactive TCRs are deleted (Robey, Annu. Rev. Immunol. 12 (1994) 265-705, Jameson, Annu. Rev. Immunol. 13 (1995) 93-126). Thus, engagement of the TCR on DP thymocytes leads to two dramatically different cell fates, either survival and further maturation (positive selection) or death by apoptosis (negative selection).

Although the zeta-chain ITAMs are not essential for the development (Shores, Science 266 (1994) 1047-1050) and MHC-restricted selection (Simpson, Int. Immunol. 7 (1995) 287-293) of mature SP T-cells in the thymus, they seem to contribute to the shaping of the T-cell repertoire by amplifying the signaling response generated by TCR-engagement during thymocyte selection.

Indeed, the results of a recent study revealed that, although no individual ITAM was specifically required, there was a direct relationship between the number of zeta-chain ITAMs within the TCR complex and the efficiency of both positive and negative

selection (Shores, J. Exp. Med. 185 (1997) 893-900). These results might be expected if positive and negative selection are dictated primarily by TCR signaling thresholds and if the magnitude of the signaling response to ligands of different affinity, critical in determining the fate of developing thymocytes, is more or less amplified by the triplicated ITAMs of the native zeta-chain or by a reduced number of ITAMs in zeta-chain mutants, respectively.

Thus it is expected, that the repertoire of positively selected thymocytes in the presence of the native zeta-chain markedly differs from that selected in the presence of a signaling deficient zeta chain derivative, and that the latter repertoire contains T-cells that might otherwise be negatively selected (Shores, Current Opinion in Immunology 9 (1997) 380-389).

NK cells are large granular lymphocytes that make up 10 to 15% of peripheral blood lymphocytes (PBL). They are capable of killing tumor cells and certain virally infected cells in a manner not restricted by the major histocompatibility complex (MHC) (Trinchieri, Adv. Immunol. 47 (1989) 187-376, Ritz, Adv. Immunol. (1988) 181-211). This cytolytic effector function does not require prior sensitization or antigen presentation by accessory cells. These properties allow NK-cells to effect an innate host defense prior to the elicitation of an antigen-specific immune response. NK-cells are known to effect two forms of cytolytic activity, natural cytotoxicity and ADCC (antibody dependent cellular cytotoxicity) that also kills target cells resistant to natural cytotoxicity.

Unlike the cytotoxic activity of T-cells that is triggered by an activation signal generated through engagement of the clonotypic TCR, natural cytotoxicity of NK-cells is primarily regulated by inhibitory signals (Yokoyama, J. Exp. Med. 186 (1997) 1803-1808). The engagement of inhibitory NK-cell receptors by specific binding to MHC class I molecules on target cells, leads to inhibition of natural cytotoxicity, that is released in the absence of target cell MHC class I expression, thus allowing the activation of natural killing. NK-cell receptors contain intracytoplasmatic immunoreceptor tyrosine-based inhibitory motifs (ITIM, consensus sequence I/V-X-Y-X-X-L) that mediate inhibitory signals after tyrosine phosphorylation induced by receptor engagement (Muta, Nature 368 (1994) 70-73, Thomas, J. Exp. Med. 181 (1995) 1953-1956).

ADCC is mediated by the low affinity IgG Fc-receptor FcγRIIIA and can be demonstrated in vitro by incubating NK-cells with antibody-coated target cells. Ligand binding and crosslinking of FcγRIIIA induce NK-cell activation resulting in cytolytic activity, up-regulation of surface activation molecules and cytokine secretion (Chehimi, J. Exp. Med. 75 (1992) 789, Ravetch, Annu. Rev. Immunol. 9 (1991) 457). FcγRIIIA is expressed as a complex comprising the transmembrane ligand-binding receptor glycoprotein CD16 and two membrane-spanning chains, gamma and zeta, which are responsible for both receptor assembly and signal transduction (Anderson, Proc. Natl. Acad. Sci. USA. 87 (1990) 2274-2278, Ravetch, Annu. Rev. Immunol. 9 (1991) 457). The gamma chain was originally identified as a subunit of the high affinity receptor for IgE and belongs to the same family of signal transduction molecules together with the zeta- and the eta-chain. Upon engagement of FcγRIIIA on NK-cells tyrosine phosphorylation occurs within the ITAMs of the zeta- and gamma-chain thus inducing further signal transduction events including the recruitment of specific Src homology (SH)2 domain containing proteins to the FcγRIIIA-complex. As a recent study demonstrated, the FcγRIIIA-mediated NK-cell activation seems to be imitated by the engagement of chimeric zeta-chain receptors transfected into NK-cells, thus resembling analogous approaches in T-cells (Tran, J. Immunol. 155 (1995) 1000-1009).

Whereas it becomes apparent from the above that antibodies specifically interacting with /recognizing the extracellular domain of the human zeta chain on the surface of intact cells were in great demand for a variety of purposes such as artificial signal transduction on T cells or NK-cells, for example, in the treatment of tumors, to date no successful experiments have been reported. The lack of success in producing an antibody that fulfills this need may primarily be due to the rather short length (9 amino acids) of this domain, possibly in conjunction with the association of the zeta-chain with the T-cell receptor and the CD3 complex on T-cells or with the IgG-Fc-receptor on NK-cells.

The technical problem underlying the present invention was therefore to provide a tool that may successfully be applied to the above-identified need. The solution to this

technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a nucleic acid molecule comprising a nucleic acid sequence encoding at least one complementary determining region (CDR) of a variable region of an antibody, said antibody specifically interacting with the extracellular domain of the human zeta-chain on the surface of intact cells, said antibody being obtainable by immunizing a rat with Jurkat cells and subsequently with a conjugate comprising a carrier molecule and a peptide comprising the 11 N-terminal amino acids of a the rat zeta-chain.
Said intact cells are preferably T-cells or NK-cells or precursors thereof, but may also be artificially transfected cells.
In accordance with the present invention, the antibody comprising said CDR is obtainable by priming rats with Jurkat (ATCC TIB-152) cells and boosting them with a conjugate comprising a carrier molecule and the above recited peptide. It is also envisaged that alternative immunization strategies may be successful such as injecting, for example, rats with one or more doses of the conjugate. Whereas in generating the antibody KLH has been used as a carrier, different carriers might be successfully employed. An example of a different carrier is BSA.

Since zeta-chain molecules occur on the surface of T-cells and NK-cells either in association with the TCR and CD3 or with Fcγ-RIIIA or possibly as free floating homo- or heterodimers, said interaction may be with either of these forms of zeta or with all of them. Similarly, the antibody may interact with a single zeta chain or specifically with the dimeric structure. Since eta has the same extracellular domain in humans as zeta, the antibody of the invention also recognizes eta in the same conformation and/or associations as the zeta-chain. Further, the antibody will also specifically interact with the extracellular domain of the zeta/eta-chains of rats and mice.

A particularly advantageous property of the antibody of the invention is the fact that it recognizes zeta/eta both on T-cells and NK-cells as well as precursors thereof. In view of what was known about the structure and associations of the zeta chain, the

development of such an antibody has indeed to be considered as surprising. Epitopes on the extremely short, 9 amino acid peptide are necessarily located very close to the cell membrane. The close structural and hence spatial association with multimolecular protein complexes on the cell surface make it seem likely, that these epitopes will also be sterically inaccessible for such a large structure as an antibody. Furthermore, due to the association of the zeta-chain with different multimolecular protein complexes on T-lymphocytes and NK-cells, extracellular zeta chain epitopes unexpectedly accessible for antibody molecules on T-cells are unlikely to be identical with those on NK-cells. In addition, due to the sequence identity in humans, rats and mice, the extracellular zeta-chain domain represents a self-antigen in all three species, thus making it unlikely to obtain specific antibodies against it by immunization of mice and rats.

The above notion that the development of this antibody must be regarded as highly surprising is corroborated by the fact that the most promising approach to obtain such an antibody failed. Namely, a combinatorial antibody library cloned from the RNA of spleen cells of mice immunized with the peptide-KLH conjugate was displayed on filamentous phage and selected in vitro by alternate panning on peptide-BSA conjugate, purified $CD8^+$-T-lymphocytes and purified NK-cells. Although enrichment of phage clones displaying Fab-antibody fragments reactive with the peptide-BSA conjugate was attained during the last panning step on $CD8^+$-T-lymphocytes, most of them were lost during the subsequent panning step on purified NK-cells, thus indicating the lack of antibodies within the repertoire that recognize a common extracellular zeta-chain epitope on T-lymphocytes and NK-cells. This was confirmed by testing a large number of clones that were reactive with the peptide-BSA conjugate, of which none could be identified with crossreactive binding activity on T-lymphocytes and NK-cells.

Only when the inventors applied an old-fashioned and rather cumbersome approach to the generation of such an antibody, they were eventually successful. This approach comprised the following steps: A peptide comprising the 11 first N-terminal amino acids of the zeta-chain was synthesized and coupled to KLH via the SH-group on cystein 11. Rats preimmunized with Jurkat cells and mice were immunized with this

conjugate, respectively, and hybridoma cell lines obtained were screened against another conjugate consisting of the 11-amino acid peptide coupled to BSA. 150 murine and 45 rat hybridoma cell lines were obtained that recognized the peptide-BSA conjugate, of which only one rat IgM antibody could be identified that exhibited binding activity to both T-lymphocytes and NK-cells as determined by flow cytometry.

The antibodies of the present invention or their corresponding immunoglobulin chain(s) can be further modified using conventional techniques known in the art, for example, by using amino acid deletion(s), insertion(s), substitution(s), addition(s), and/or recombination(s) and/or any other modification(s) known in the art either alone or in combination. Methods for introducing such modifications in the DNA sequence underlying the amino acid sequence of an immunoglobulin chain are well known to the person skilled in the art; see, e.g., Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. The antibody may also be a chimeric antibody.

Since it was well known in the art that recognition of an epitope is often governed by a single CDR, preferably the CDR of the heavy chain, it is envisaged that one CDR of the antibody obtainable according to the above outlined schedule will be sufficient to contribute to at least a weak but significant binding. This holds preferably true for the antibody that was actually obtained by the above referenced strategy. Preferably, however, said nucleic acid molecule comprises a nucleic acid sequence encoding at least two CDRs of said variable region.

In another preferred embodiment of the nucleic acid molecule of the invention, said nucleic acid molecule comprises a nucleic acid sequence encoding three CDRs of said variable region.

A further preferred embodiment of nucleic acid molecule of the invention is characterized in that said nucleic acid sequence encodes a V_H chain.

In another preferred embodiment of nucleic acid molecule the invention, said nucleic acid sequence encodes a V_L chain.

The nucleic acid molecule of the invention may, for example, be an RNA molecule or a DNA molecule. In an additional preferred embodiment the nucleic acid molecule of the invention is a DNA molecule. Particularly preferred is a synthetic or semisynthetic DNA molecule.

In a further preferred embodiment of the nucleic acid molecule of the invention said CDR has one of the following nucleotide sequences:

(a) SEQ ID No. 1
(b) SEQ ID No. 3
(c) SEQ ID No. 5
(d) SEQ ID No. 7
(e) SEQ ID No. 9
(f) SEQ ID No. 11

In a particularly preferred embodiment of the nucleic acid molecule of the invention said V_H-chain has the nucleotide sequence of SEQ ID No. 13 or encodes the amino acid sequence of SEQ ID No. 14.

In another particularly preferred embodiment of the nucleic acid molecule of the invention said V_L-chain has the nucleotide sequence of SEQ ID No. 15 or encodes the amino acid sequence of SEQ ID No. 16.

The invention also relates to the nucleic acid molecule of any one of claims 1 to 6 wherein the CDR encodes one of the amino acids sequences of SEQ ID Nos. 2, 4, 6, 8, 10 or 12.

The invention also relates to a vector comprising the nucleic acid molecule of the invention.

The vector of the invention may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions. Preferably, the polynucleotide of the invention is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic cells.

Expression of said polynucleotide comprises transcription of the polynucleotide into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They usually comprise regulatory sequences ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers, and/or naturally-associated or heterologous promoter regions. In this respect, the person skilled in the art will readily appreciate that the polynucleotides encoding at least the variable domain of the light and/or heavy chain may encode the variable domains of both immunoglobulin chains or only one. Likewise, said polynucleotides may be under the control of the same promoter or may be separately controlled for expression. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the PL, lac, trp or tac promoter in E. coli, and examples for regulatory elements permitting expression in eukaryotic host cells are the AOX1 or GAL1 promoter in yeast or the CMV-, SV40- , RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. Furthermore, depending on the expression system used leader sequences capable of directing the polypeptide to a cellular compartment or secreting it into the medium may be added to the coding sequence of the polynucleotide of the invention and are well known in the art. The leader sequence(s) is (are) assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein, or a portion thereof, into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an C- or N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), or pSPORT1 (GIBCO BRL).

Preferably, the expression control sequences will be eukaryotic promoter systems in

vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the immunoglobulin light chains, heavy chains, light/heavy chain dimers or intact antibodies, binding fragments or other immunoglobulin forms may follow; see, Beychok, Cells of Immunoglobulin Synthesis, Academic Press, N.Y., (1979).

The vector of the present invention which may e.g. be a plasmid, cosmid, virus or bacteriophage is preferably an expression vector. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the polynucleotides or vector of the invention into targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the polynucleotides and vectors of the invention can be reconstituted into liposomes for delivery to target cells. The vectors containing the polynucleotides of the invention (e.g., the heavy and/or light variable domain(s) of the immunoglobulin chains encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts; see Sambrook, supra.

The invention further relates to a host transformed or transfected with the vector of invention.

Said host cell may be a prokaryotic or eukaryotic cell. The polynucleotide or vector of the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained extrachromosomally.

The host cell can be any prokaryotic or eukaryotic cell, such as a bacterial, insect, fungal, plant, animal or human cell. Preferred fungal cells are, for example, those of the

genus Saccharomyces, in particular those of the species S. cerevisiae. The term "prokaryotic" is meant to include all bacteria which can be transformed or transfected with a DNA or RNA molecules for the expression of an antibody of the invention or the corresponding immunoglobulin chains. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, E. coli, S. typhimurium, Serratia marcescens and Bacillus subtilis. The term "eukaryotic" is meant to include yeast, higher plant, insect and preferably mammalian cells. Depending upon the host employed in a recombinant production procedure, the (poly)peptides/antibodies or immunoglobulin chains encoded by the polynucleotide of the present invention may be glycosylated or may be non-glycosylated. Antibodies of the invention or the corresponding immunoglobulin chains may also include an initial methionine amino acid residue. A polynucleotide of the invention can be used to transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Furthermore, methods for preparing fused, operably linked genes and expressing them in, e.g., mammalian cells and bacteria are well-known in the art (Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). The genetic constructs and methods described therein can be utilized for expression of the (poly)peptide/antibody of the invention or the corresponding immunoglobulin chains in eukaryotic or prokaryotic hosts. In general, expression vectors containing promoter sequences which facilitate the efficient transcription of the inserted polynucleotide are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of the transformed cells. Furthermore, transgenic animals, preferably mammals, comprising cells of the invention may be used for the large scale production of the (poly)peptide of the invention.

The transformed hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. Once expressed, the whole (poly)peptides/antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention, can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like; see, Scopes, "Protein Purification", Springer-Verlag, N.Y. (1982). The antibody or its corresponding immunoglobulin chain(s) of the invention can then be isolated from the growth medium, cellular lysates, or cellular

membrane fractions. The isolation and purification of the, e.g., microbially expressed antibodies or immunoglobulin chains of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibodies directed, e.g., against the constant region of the antibody of the invention. It will be apparent to those skilled in the art that the antibodies of the invention can be further coupled to other moieties for, e.g., drug targeting and imaging applications. Such coupling may be conducted chemically after expression of the (poly)peptide/antibody or antigen to site of attachment or the coupling product may be engineered into the (poly)peptide/antibody of the invention at the DNA level. The DNAs are then expressed in a suitable host system, and the expressed proteins are collected and renatured, if necessary.

The invention relates further to a method of producing a (poly)peptide encoded by the nucleic acid molecule of the invention comprising culturing the host of the invention under suitable conditions and isolating said (poly)peptide from the culture.
Culturing of said host cells is, in general, described above and may be effected according to established protocols. The same holds true for the isolation of the (poly)peptides.

Additionally, the invention relates to a (poly)peptide that is encoded by the nucleic acid molecule of invention or produced by the method of the invention.

The invention also relates to an antibody or fragment or derivative thereof comprising at least one (poly)peptide of the invention.
Preferably, the antibody of the invention comprises both the complete above-referenced V_H and V_L chains in conjunction with appropriate constant regions such as μ, γ, α, κ or λ chain.

Specific applications of the antibody or fragment or derivative of the invention include the following:
Mature T-lymphocytes may be functionally affected by structural or functional TCR-blockage as a result of antibodies or antibody derivatives specifically bound to the

zeta-chain or by virtue of biologically or pharmaceutically active molecules targeted to the T-cell surface by anti-zeta chain antibodies or antibody fragments/derivatives.
Furthermore, development and selection of thymocytes may be affected by structural or functional TCR- or pre-TCR-blockage as a result of antibodies or antibody fragments/derivatives specifically bound to the zeta chain.
As the zeta chain is consistently expressed during the whole T-cell development from the most immature thymocytes to the mature T-lymphocytes the molecule may be useful for targeting a broad range of T-cell malignancies.
NK-cells may also be functionally affected by structural or functional FcγRIIIA-blockage as a result of antibodies or antibody derivatives specifically bound to the zeta-chain or by virtue of biologically or pharmaceutically active molecules targeted to the NK-cell surface by anti-zeta chain antibodies or antibody fragments or derivatives thereof.

The antibody or fragments or derivatives thereof may, inter alia, be a (semi)synthetic or a classically developed monoclonal antibody. Fragments include Fab' or $F(ab)_2$ fragments.

The antibodies of the present invention can comprise a further domain, said domain being linked by covalent or non-covalent bonds. The linkage can be based on genetic fusion according to the methods known in the art and described above or can be performed by, e.g., chemical cross-linking as described in, e.g., WO 94/04686. The additional domain present in the fusion protein comprising the antibody of the invention may preferably be linked by a flexible linker, advantageously a polypeptide linker, wherein said polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of said further domain and the N-terminal end of the antibody of the invention or vice versa. The above described fusion protein may further comprise a cleavable linker or cleavage site for proteinases. These spacer moieties, in turn, can be either insoluble or soluble (Diener, et al., Science, 231:148, 1986) and can be selected to enable drug release from the antigen at the target site. Examples of therapeutic agents which can be coupled to the antibodies of the invention for immunotherapy are drugs, radioisotopes, lectins, and toxins. The drugs with which can be conjugated to the

antibodies of the invention include compounds which are classically referred to as drugs such as mitomycin C, daunorubicin, and vinblastine.

In using radioisotopically conjugated antibodies of the invention for, e.g., immunotherapy, certain isotopes may be more preferable than others depending on such factors as leukocyte distribution as well as stability and emission. Depending on the autoimmune response, some emitters may be preferable to others. In general, α and β particle-emitting radioisotopes are preferred in immunotherapy. Preferred are short range, high energy α emitters such as ^{212}Bi. Examples of radioisotopes which can be bound to the antibodies, antigens or epitopes of the invention for therapeutic purposes are ^{125}I, ^{131}I, ^{90}Y, ^{67}Cu, ^{212}Bi, ^{212}At, ^{211}Pb, ^{47}Sc, ^{109}Pd and ^{188}Re. Other therapeutic agents which can be coupled to the antibody, antigen or epitope of the invention, as well as ex vivo and in vivo therapeutic protocols, are known, or can be easily ascertained, by those of ordinary skill in the art. Wherever appropriate the person skilled in the art may use a polynucleotide of the invention encoding any one of the above described antibodies, antigens or epitopes or the corresponding vectors instead of the proteinaceous material itself.

In a preferred embodiment, the antibody of the invention is a monoclonal antibody.

In another preferred embodiment of the invention the antibody the invention is a bispecific antibody.

In a preferred embodiment of the bispecific antibody of the invention, the first specificity is for the extracellular domain of the human zeta-chain on the surface of an intact cell and the second specificity is for an optionally different molecule on the surface of a T-lymphocyte, a natural killer cell or a precursor thereof.

The bispecific antibody of the invention may bind to the above-referenced targets which may be located on the same cell or on different cells. Said different cells may be, for example, two different T-lymphocytes of the same type or a T-lymphocyte and a natural killer cells or precursors of the above, respectively.

In another preferred embodiment of the bispecific antibody of the invention the first specificity is for the extracellular domain of the human zeta-chain on the surface of an

intact cell and the second specificity is for a different molecule on the surface of a different cell, preferably of a cell different from a T-cell, an NK-cell or a precursor thereof. Preferably this molecule is a virus encoded antigen, a tumor associated antigen or a surface antigen either on antigen presenting cells (APCs), most preferably dendritic cells, or on non-APCs.

A preferred application of the bispecific antibody of the invention is to redirect T-lymphocytes against target cells by simultaneously targeting the zeta chain and a target cell surface antigen with a bispecific antibody instead of directing T-lymphocytes on target cells by transfecting them with a chimeric zeta-chain receptor (Romeo, Cell 64 (1991) 1037-1046). Since the bispecific antibody binds to native zeta chain, which is associated with the other TCR-subunits, the signaling machinery of the whole TCR-complex may thus be recruited. In contrast, chimeric zeta-chain receptors do not associate with endogenous TCR-subunits and may thus only recruit the isolated signaling effect of the zeta-chain, which seems to be insufficient to activate resting T-cells or may cause unbalanced alteration of the functional state of T-lymphocytes with respect to the induction of activation versus apoptosis.

A preferred application of T-cell retargeting comprises the lysis of target cells by directing on them the cytotoxic activity of cytotoxic T-lymphocytes. Another preferred application of T-cell retargeting comprises the priming of naive T-lymphocytes by crosslinking of their zeta-chain molecules with a surface antigen on antigen presenting cells (APC) or on non-APCs which have been modified to provide sufficient costimulatory signals. On the other hand naive T cells may be anergized or depleted by zeta-chain mediated retargeting on cells that do not provide sufficient costimulatory signals.

Another preferred application of T-cell retargeting comprises the induction of apoptosis in mature activated T-lymphocytes by strong zeta chain mediated TCR reengagement thus imitating mechanisms that mediate peripheral T-cell tolerance and contribute to peripheral immune homeostasis.

Thymic selection of thymocytes during T-cell development may be modified by bispecific antibodies through crosslinkage of the zeta chain on thymocytes with surface molecules on thymic antigen presenting cells directly involved in the positive and negative selection process.

A further preferred application of the bispecific antibody of the invention comprises the retargeting of the cytotoxic activity of NK-cells against target cells by simultaneously targeting the zeta-chain and a target cell surface antigen with a bispecific antibody instead of directing NK-cells on target cells by transfection of a chimeric zeta-chain receptor (Tran, J. Immunol. 155 (1995) 1000-1009).

In a further preferred embodiment the derivative of the antibody of the invention is an scFv chain.

In a preferred embodiment the monoclonal antibody of the invention, said antibody is an IgM.

The invention relates further to a bispecific receptor comprising a (poly)peptide of the invention and natural receptor, natural ligands or a derivative thereof interacting with a surface molecule on the same or on another cell; preferably said receptors or ligands are CD4, CTLA-4, B7-1, B7-2, LFA-3, ICAM-1, -2, -3 or chemokines like MIP-1α, MIP-1β, RANTES or SDF-1.
Applications of the bispecific antibody of the invention are envisaged to also apply for the bispecific receptor of the invention.

The invention relates further to a pharmaceutical composition comprising the nucleic acid molecule of the invention, the vector, the host, the (poly)peptide, the antibody or fragment or derivative thereof and/or the bispecific receptor of the invention.

The pharmaceutical composition of the present invention may further comprise a pharmaceutically acceptable carrier. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be

administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. A typical dose can be, for example, in the range of 0.001 to 1000 µg (or of nucleic acid for expression or for inhibition of expression in this range); however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{12} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the pharmaceutical composition of the invention may comprise further agents such as interleukins or interferons depending on the intended use of the pharmaceutical composition.

It is envisaged by the present invention that the various polynucleotides and vectors of the invention are administered either alone or in any combination using standard vectors and/or gene delivery systems, and optionally together with a pharmaceutically acceptable carrier or excipient. Subsequent to administration, said polynucleotides or vectors may be stably integrated into the genome of the subject. On the other hand, viral vectors may be used which are specific for certain cells or tissues and persist in said cells. Suitable pharmaceutical carriers and excipients are well known in the art.

Furthermore, it is possible to use a pharmaceutical composition of the invention which comprises polynucleotide or vector of the invention in gene therapy. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses, and adeno-associated viruses, among others; see also supra. Delivery of nucleic acids to a specific site in the body for gene therapy may also be accomplished using a biolistic delivery system, such as that described by Williams (Proc. Natl. Acad. Sci. USA 88 (1991), 2726-2729).

The pharmaceutical compositions, methods and uses of the present invention may be desirably employed in humans, although animal treatment is also encompassed by the methods and uses described herein.

The invention also relates to the use of the antibody of the invention wherein the first specificity is for the extracellular domain of the human zeta-chain and the second specificity is for a different molecule on the surface of a T-lymphocyte, a natural killer cell or a precursor thereof for the preparation of a pharmaceutical composition for the treatment or prevention of autoimmune diseases, immune deficiencies, T-cell malignancies, infectious diseases or for the suppression of immune response especially in order to avoid graft rejection after organ transplantation.
Beside the elimination of target cells (e.g. tumor cells or virus infected cells) the modes described in the present invention of engaging (normal or malignant) cells of the T-cell lineage by extracellular targeting of the zeta chain may be therapeutically used to enhance or suppress immune responses and/or to influence T-cell disorders

related to autoimmune diseases, immunodeficiencies or T-cell malignancies. Immune suppression based on the extracellular targeting of the zeta- (and eta-) chain may be preferably used to prevent graft rejection after transplantation.
The modes described in the present invention of modifying signal transduction during T cell development and thymocyte selection by extracellular targeting of the zeta chain may be therapeutically used to enhance favorable immune responses e.g. in case of infectious diseases, tumors and immunodeficiencies or to suppress unfavorable immune responses e.g. in case of autoimmune diseases or graft rejection after transplantation.

Further, the invention relates to the use of the antibody of the invention wherein the first specificity is for the extracellular domain of the human zeta-chain and the second specificity is for a different molecule on the surface of a different cell for the preparation of a pharmaceutical composition of the treatment or prevention of malignancies, viral infections and other infectious diseases.

The invention in addition relates to the use of the (poly)peptide or the antibody or fragment or derivative thereof or the bispecific receptor of the invention for the preparation of a pharmaceutical composition for the enhancement or suppression of NK-cell dependent immunity or for the treatment of NK-cell derived malignancies.
Beside the elimination of target cells (e.g. tumor cells or virus infected cells) the described modes of engaging NK-cells by extracellular targeting of the zeta chain may be therapeutically used to enhance or suppress NK-cell dependent immunity or to influence NK-cell derived malignancies. As the zeta-chain is expected to be also expressed on NK-cell derived malignancies, the molecule may furthermore be useful for targeting malignant NK-cells.

In addition the invention relates to a method for the determination of zeta-chain or eta-chain expression on NK-cells, T-lymphocytes or precursors thereof comprising

(a) contacting the (poly)peptide or the antibody or fragment or derivative thereof of the invention with said NK-cells, T-lymphocytes or precursors thereof; and

(b) assessing the amount of bound (poly)peptide, antibody or derivative.

Contacting can be carried out by incubating preferably on ice said (poly)peptide or said antibody or fragment or derivative thereof with said NK-cells, T-lymphocytes or precursors thereof in a biological buffer resembling physiological conditions (e.g. phosphate-buffered saline, PBS) for 20 to 40 minutes. After two washing steps with PBS bound (poly)peptide, antibody or fragment or derivative thereof can be detected for example with an appropriate fluorescent-labeled secondary antibody and quantitated by flow cytometric analysis as described in Example 4.

The invention relates further to a kit comprising the nucleic acid molecule, the vector, the host, the (poly)peptide, the antibody or fragment or derivative thereof and/or the bispecific receptor of the invention.

Finally, the invention relates to a transgenic animal comprising in its germline at least one copy of the nucleic acid molecule or the vector of the invention.
Transgenic animals may be produced according to conventional protocols as described, for example in Palmiter R.D., Brinster R.L.: Germline transformation of mice. Ann. Rev. Genet. 20 (1986), 465-499 and Capecci M.: Altering the genome by homologous recombination. Science 244 (1991) 1288-1292. Preferred examples of the transgenic animals of the invention are cows, sheep, rabbits, mice or rats.

Table 1 shows primer sets for the PCR-amplification of murine Ig-heavy and light chain-DNA-fragments

The figures show:

Figure 1: Structure of the TCR and early events in T-cell activation. The TCR consists of clonotypic chains ($\alpha + \beta$) and invariant chains (ζ, CD3γ, CD3δ, and CD3ε), with the probable subunit composition TCR $\alpha + \beta$, CD3 $\varepsilon\delta\gamma\varepsilon$, $\zeta\zeta$. The location of the ITAMs within the cytoplasmatic domains of the CD3 and ζ chains are shown as small black ovals. **A)** In resting T-cells, ITAMs are either non-phosphorylated or only

partially phosphorylated. The protein tyrosine kinase Lck, is associated with the cytoplasmic domain of CD4 or CD8. **B)** Upon interaction with an MHC-peptide-complex, the TCR and either CD4 or CD8 are co-aggregated and the ITAMs within the CD3 and ζ chains are phosphorylated by the Src kinases Lck and/or Fyn. ZAP-70 or the related kinase, Syk, binds specifically to ITAMs in which both tyrosine residues have been phosphorylated. Following its recruitment to the TCR-complex, ZAP-70 is activated by Lck, subsequently after other molecules are thus recruited to the TCR-complex and activation proceeds to further downstream molecules (not shown). Circled P represents phosphorylated tyrosine residues.

Figure 2 : ELISA-analysis of murine Fab-antibody-fragments selected by phage display for binding to the extracellular part of the human zeta-chain. Periplasma preparations of soluble Fab-fragments expressed in E. *coli* were incubated with immobilized zeta-peptide-BSA-conjugate. Specifically bound Fab-fragments were detected with a horse radish peroxidase conjugated $F(ab')_2$ fragment of a goat anti-mouse IgG + IgM antibody. The ELISA was developed by adding an ABTS-substrate solution. Eight clones per round of in vitro selection are presented on the x-axis; OD-values were measured by an ELISA-reader at 405 nm and are presented on the y-axis. For negative controls, the wells were incubated with PBS instead of periplasma preparations.

Figure 3: Flowcytometry-analysis of the zeta-chain specific binding activity of the 2-B-5 antibody on the surface of $CD8^+$-T-lymphocytes and NK-cells. 100.000 mononucleated cells from peripheral blood of two different healthy donors were incubated with undiluted cell culture supernatant of the 2-B-5 hybridoma. Bound zeta-chain specific rat antibody was detected by a fluorescein (FITC) conjugated goat-anti-rat Ig (IgG + IgM) antibody diluted 1:100 in PBS. Triple color fluorescence analysis was carried out by applying a positive gate for $CD8^+$ (Tricolor) and a negative gate for $CD16^+$ (PE) cells thus allowing the detection of FITC-mediated fluorescence (filled lines) exclusively attributed to $CD8^+$-T-lymphocytes (phenotype: $CD8^+$, $CD16^-$) without any contaminating signals from $CD8^+$-NK-cells. Similarly, triple color fluorescence analysis was carried out by applying a positive gate for $CD56^+$- (PE) and a negative gate for $CD3^+$-cells (tricolor) thus allowing the detection of FITC-mediated

fluorescence (filled lines) exclusively attributed to NK-cells (phenotype: $CD56^+$, $CD3^-$) without any contaminating signals from $CD56^+$-T-lymphocytes. As isotype control (broken lines) culture supernatant of an antibody with the same isotype (rat IgM) but irrelevant specificity was used. For the fixation of labeled cells 1% paraformaldehyde in PBS was used. Cells were analyzed by flowcytometry on a FACS-scan (Becton Dickinson).

Figure 4: Results of a Sandwich-ELISA confirming the reactivity of antibody 2-B-5 with native zeta-chain present in the lysate of the $CD8^+$-T-cell lymphocytes. A zeta-chain specific antibody, which recognizes the amino acids 144-163 at the carboxy-terminus of the human zeta chain was coated for 12 hours to the wells of a 96 U-bottom plate, followed by blockade for one hour at room temperature with PBS/ 3% BSA. Subsequently the lysate of $CD8^+$-cells was added undiluted and in several dilutions and incubated for one hour at room temperature. As negative control, the wells were incubated with PBS instead of the cell lysate. In the following step the purified antibody 2-B-5 was added at a concentration of 1 µg/ml and incubated for one hour. Bound 2-B-5 antibody was detected with a biotinylated mouse-anti-rat IgM antibody followed by an Avidin-peroxidase-conjugate. The ELISA was finally developed by addition of ABTS-substrate solution. The colored precipitate was measured at 405 nm using an ELISA-reader.

Figure 5: ELISA-based analysis for specific binding to zeta-peptide-KLH-conjugate of recombinant Fab-fragment of the rat monoclonal antibody 2-B-5 expressed in the periplasma of E. *coli*. Coating of zeta-peptide-KLH-conjugate was carried out at 4°C for 12 hours followed by a single washing step with PBS/0,05% Tween. The wells were blocked for 1 hour with PBS/3% bovine serum albumin (BSA) and washed again once. Then Fab-containing periplasma preparations were added undiluted and in several dilutions and incubated for 2 hours. As negative controls, wells were incubated with PBS instead of periplasma preparations. For detection of Fab-fragments bound to the zeta-peptide-KLH-conjugate a murine anti-His-tag antibody was used followed by a peroxidase conjugated polyclonal goat anti-mouse IgG antibody. The ELISA was finally developed by addition of ABTS-substrate

solution. The turnover of colored substrate was measured by an ELISA-Reader at OD 405 nm.

Figure 6: DNA- and protein-sequence of the VH-region of the anti-zeta-chain antibody 2-B-5. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are presented in single letter code. Boxes indicate the three CDR's.

Figure 7: DNA- and protein-sequence of the VK-region of the anti-zeta-chain antibody 2-B-5. Numbers indicate the nucleotide (nt) positions, amino acids (aa) are presented in single letter code. Boxes indicate the three CDR's.

Figure 8: Results of an ELISA-based BrdU-incorporation-assay detecting cell proliferation carried out in order to determine the stimulation of $CD8^+$-T-cells, NK-cells and PBMC induced by the anti-zeta-chain-antibody 2-B-5. A 96-well flat-bottom microtiterplate was coated with purified 2-B-5 antibody in several dilutions overnight at 4°C. 100.000 $CD8^+$-T-lymphocytes, NK-cells and unseparated PBMC were added in triplicates to the wells of a microtiterplate, respectively. To control the specificity of the stimulation mediated by 2-B-5 an antibody of the same isotype (rat IgM) with irrelevant specificity was used at the same concentrations. The antibody OKT3 (Isotype IgG2a), which recognizes the human CD3-complex was applied as specific positive control for the stimulation of T-cells and unseparated PBMC, respectively. A murine IgG2a-antibody of irrelevant specificity was used as isotype control for OKT3. A blank control (wells without cells) and a background control (wells without BrdU) were also included. After incubation period of three days the BrdU-labeling solution was added for 24 hours. Subsequently cells were lysed and fixed followed by the addition of an anti-BrdU-antibody, which binds to the BrdU incorporated in newly synthesized, cellular DNA. This peroxidase conjugated antibody was detected by the subsequent substrate reaction. The reaction product was quantified by an ELISA reader at a wavelength of 450 nm.

Figure 9: Flowcytometric analysis of TCR/CD3 complex internalization induced by the binding of the anti-zeta-chain antibody. 200.000 mononucleated cells were incubated with the anti-zeta-chain antibody 2-B-5 at a concentration of 1 µg/ml, at 4°C

or 37°C for either 30 or 60 minutes; a parallel experiment was carried out with a rat-anti-human CD3 antibody. The capping process was terminated by washing twice with cold PBS. To label the cell surface bound antibody, the cells were incubated with a fluorescein (FITC) conjugated goat-anti-rat Ig (IgG + IgM) antibody diluted 1:100 in PBS. As negative control, only the secondary antibody was used.

Figure 10: DNA- and protein-sequence of the anti-zeta-chain/anti-EpCAM bispecific single-chain antibody. Numbers indicate the nucleotide (nt) positions, the resulting amino acid sequence is depicted below the nucleotide sequence. The DNA sequence encoding the antibody starts at position 67 and ends at position 1605. Nucleotides 10 to 66 encode a leader peptide, that mediates secretion of the bispecific antibody in mammalian cells. The first six nt (position 1 to 6) and the last six nt (position 1632 to 1637) contain the restriction enzyme recognition sites for EcoRI and SalI, respectively.

Figure 11: Cytotoxic activity of PBMC and CD8+-T-lymphocytes redirected against EpCAM-positive Kato cells by the bispecific anti-zeta-chain/anti-EpCAM antibody. 200.000 unstimulated PBMCs or CD8+ T-lymphocytes in a volume of 100 µl were added to 10.000 Chromium-51 labeled Kato III cells in a volume of 100 µl. The bispecific antibody was added in concentrations from 40 ng/ml to 5 µg/ml in a volume of 50 µl. The microtiterplates were incubated for 16 h at 37°C, 5 % CO2. After the incubation period 50 µl supernatant were removed from each well and assayed for released 51Cr in a gamma counter.

Figure 12: Cytotoxic activity of NK-cells redirected against EpCAM-positive target cells by the bispecific anti-zeta-chain/anti-EpCAM antibody. 100.000 NK-cells in a volume of 100 µl were added to 10.000 Chromium-51 labeled Kato III cells in a volume of 100 µl. The bispecific antibody was added in a concentration of 1 µg/ml in a volume of 50 µl. The microtiterplates were incubated for 4 h at 37°C, 5 % CO2. After the incubation period 50 µl supernatant were removed from each well and assayed for released 51Cr in a gamma counter.

These and other embodiments are disclosed and encompassed by the description and Examples of the present invention. Further literature concerning any one of the antibodies, methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries and databases, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

The examples illustrate the invention.

Example 1: Immunization of mice with zeta-peptide-KLH-conjugate and determination of serum titer using zeta-peptide-BSA-conjugate

Ten weeks old F1 mice from balb/c x C57black crossings were immunized with the zeta-peptide-KLH–conjugate (Jerini Bio Tools, Berlin). The peptide, with the amino acid sequence (QSFGLLDPKLC) of the zeta-chain N-terminus was coupled to the maleinimide activated KLH in directed manner via the mercapto-group of the C-terminal Cystein. The conjugate was dissolved in 0.9% NaCl at a concentration of 100 µg/ml. The solution was subsequently emulsified 1:2 with complete Freund's adjuvants and 50 µl were injected per mouse intraperitonially. Mice received booster

immunizations after 4, 8, and 12 weeks in the same way, except that complete Freund's adjuvants was replaced by incomplete Freund's adjuvants. Ten days after the first booster immunization, blood samples were taken and antibody serum titer against zeta-peptide-BSA-conjugate was tested by ELISA. Serum titer was more than 1000 fold higher in immunized than in not immunized animals. Three days after the second boost, spleen cells were fused with P3X63Ag8.653 cells (ATCC CRL-1580) to generate hybridoma cell lines following standard protocols as described in Current Protocols in Immunology (Coligan, Kruisbeek, Margulies, Shevach and Strober, Wiley-Interscience, 1992). After PEG-fusion, cells were seeded at 100.000 cells per well in microtiterplates and grown in 200 µl RPMI 1640 medium supplemented with 10% fetal bovine serum, 300 units/ml recombinant human interleukin 6 and HAT-additive for selection. Culture supernatants from densely grown wells were tested by ELISA-analysis at 1:20 dilution. The ability of the hybridoma supernatants to bind the zeta-peptide-BSA-conjugate was tested by the following ELISA:

100 µl zeta-peptide conjugated to bovine serum albumin in the same way as described for the zeta-peptide-KLH-conjugate (Jerini Bio Tools, Berlin) was coated to wells of a 96 U-bottom plate (Nunc, maxisorb) at a concentration of 5 µg/ml. Coating was performed overnight at 4°C, after washing three times with washing buffer (0.1M NaCl, 0.05M Na_2HPO_4 pH 7.3, 0.05% Tween 20, 0.05% NaN_3) the following blockade was performed with 200 µl of 2% skimmed milk powder added to the washing buffer for one hour at room temperature. In the next step the hybridoma supernatant was incubated pure and at several dilutions for two hours at room temperature. As detection system diluted horseradish peroxidase conjugated polyclonal antibody against mouse immunoglobulin was used. After 5 times of washing the ELISA was finally developed by addition of TMB-substrate solution (Tetramethylbenzidine, Boehringer Mannheim). The colored precipitate was measured after 15 min. at 405 nm using an ELISA-reader.

Supernatants from 150 clones exhibiting strong ELISA-signals were selected for flowcytometric analysis.

To check the binding activity of the hybridoma supernatants on T-lymphocytes and NK-cells a flowcytometric analysis was performed. 1 x 10^6 PBMC were incubated for

30 min. on ice in 50 µl undiluted supernatant from 150 different clones, respectively and bound antibodies were detected subsequently by a fluorescein (FITC) conjugated $F(ab')_2$ fragment of a rabbit anti-mouse Ig antibody (Dako Hamburg, Code No. F0313) diluted 1:100 in PBS. To avoid unspecific binding in the following labeling steps the free valences of the FITC-conjugated antibody were blocked by addition of 50 µl 1:10-diluted mouse serum (Sigma immunochemicals, Deisenhofen, M-5905) for 30 minutes. To distinguish the two PBMC-subsets the previously labeled cells were divided. One half was stained with a 1:100 diluted tricolor conjugated anti-CD8 antibody (Caltac Laboratories; Burlingame; USA, Code No. MHCD0306); the other half was stained with a 1:25 diluted phycoerythrin (PE) conjugated anti-CD56 antibody (Becton Dickinson, Heidelberg, Cat. No. 347747). As negative control murine monoclonal antibody of irrelevant specificity was used instead of the hybridoma supernatants. Unlabeled anti-CD16 and anti-CD6 antibodies for specific staining of NK-cell or T-lymphocytes respectively were used to control the primary labeling step.

Cells were analyzed by flowcytometry on a FACS-scan (Becton Dickinson, Heidelberg). FACS-staining and measuring of the fluorescence intensity were performed as described in Current Protocols in Immunology (Coligan, Kruisbeek, Margulies, Shevach and Strober, Wiley-Interscience, 1992)

Two-color fluorescence analysis was carried out by applying a positive gate for $CD8^+$- and $CD56^+$-cells, respectively thus allowing the detection of FITC-mediated fluorescence separately on $CD8^+$-T-lymphocytes and NK-cells. Despite a clear staining of $CD8^+$-T-lymphocytes and NK-cells by the respective positive control antibodies, none of the 150 hybridoma supernatants showed binding activity on $CD8^+$-T-lymphocytes and/or NK-cells.

Example 2: In vitro selection for anti-zeta-antibodies of a murine combinatorial antibody library by the phage display method

An F1 mouse from balb/c x C57black crossings was immunized of the age of ten weeks as described in Example 1. Ten days after the first booster immunization blood samples were taken and antibody serum titer against zeta-peptide-BSA-conjugate was tested by ELISA (see Example 1). Serum titer was more than 1000 fold higher in the immunized compared to not immunized animals. Three days after the third injection, murine spleen cells were harvested. For isolating total RNA a protocol according to Chomczynski (Analytical biochemistry 162 (1987) 156-159) was used.
A DNA-library encoding murine immunoglobulin (Ig) kappa light chains and Ig heavy chain Fd-fragments (=VH+CH1) was constructed by RT-PCR on murine spleen RNA, respectively. cDNA was synthesized according to standard protocols (Sambrook, Cold Spring Harbor Laboratory Press 1989, second edition).
The primer sets (depicted in Table 1) were chosen, giving rise to a 5'- *XhoI* and a 3'-*SpeI* recognition site for the heavy chain- and to a 5'-*Sac*I and a 3'- *Xba*I recognition site for the light chain fragments. For the PCR-amplification of the HC DNA-fragments eight different 5'-VH-family specific primers were each combined with four 3' primer hybridizing to the 3´-region of the HC-CH1-domain of different IgG-subclasses; for the PCR-amplification of the kappa light chain fragments seven different 5'-VK-family specific primers were each combined with one 3'-primer, hybridizing to the 3´-end of the kappa constant region (CK).
The following PCR program was used for amplification: Initial denaturation at 94°C for 2 min.; 40 cycles of amplification: Denaturation at 94°C for 20 sec.; primer annealing at 52°C for 50 sec. and primer extension at 72 °C for 60 sec., followed by a 10 min. final extension at 72°C.

450 ng of the kappa light chain fragments (*Sac*I-*Xba*I digested) were ligated with 1400 ng of the phagmid pComb3H (*Sac*I-*Xba*I digested; large fragment) (Barbas et al, Proc Natl Acad Sci USA 88, 7978-82 (1991)). The resulting light chain library was then transformed into 300 µl of electrocompetent *Escherichia coli* XL1 Blue by electroporation (2.5 kV, 0.2 cm gap cuvette, 25 FD, 200 Ohm, Biorad gene-pulser) resulting in a library size of 6×10^8 independent clones. After one hour of phenotype

expression, positive transformants were selected for the vector encoded carbenicilline resistance in 100 ml of liquid SB-culture over night. Cells were then harvested by centrifugation and plasmid preparation was carried out using a commercially available plasmid preparation kit (Qiagen).

2800 ng of this plasmid-DNA containing the kappa light chain library (*Xho*I-*Spe*I digested; large fragment) were ligated with 900ng of the HC-Fd-DNA-fragments (*Xho*I-*Spe*I digested) and again transformed into two 300 µl aliquots of electrocompetent *E. coli* XL1 Blue by electroporation (2.5 kV, 0.2 cm gap cuvette, 25 FD, 200 Ohm) resulting in a combinatorial library of antibody Fab-fragments consisting of 4×10^8 independent clones.

After one hour of phenotype expression, positive transformation was selected by carbenicilline resistance. After this adaptation these clones were infected with an infectious dose of 1×10^{12} particles of the helper phage VCSM13 resulting in the production and secretion of filamentous M13 phage, with each Phage particle containing a single-stranded copy of the phagemid vector encoding a single murine antibody Fab-fragment and displaying the corresponding Fab-protein on the phage surface. Fab-fragments were anchored on the phage surface by an translational fusion of the HC-Fd-fragment to the phage coat protein III, with the Ig-light chain spontaneously associating with the HC-fragment.

This phage library carrying the cloned Fab-repertoire was harvested from the culture supernatant by PEG8000/NaCl precipitation and centrifugation, redissolved in RPMI 1640 – medium supplemented with 10% FCS and incubated either with immobilized zeta-peptide-BSA-conjugate or with isolated $CD8^+$-lymphocytes or NK-cells in daily alternating order. The two subpopulations of human PBMC were isolated in advance by a immunomagnetic separation method. Mononucleated cells obtained from peripheral blood by Ficoll-density gradient centrifugation were first incubated with a specific primary antibody of murine origin directed against human CD8 or CD56 and subsequently subjected to rosettation with paramagnetic beads (Dynal, Oslo, Norway) conjugated with a sheep-anti-mouse IgG antibody. $CD8^+$-T-cells and $CD56^+$-NK-cells were isolated with a magnet attached to the wall of the tube containing the cell suspension. After incubation of the phage library with purified $CD8^+$-T-lymphocytes or

NK-cells for two hours at 4°C under continuous agitation respectively, cell bound phage particles were rescued from unbound phage particles via the paramagnetic beads still attached to the cells.

Accordingly, exposure to the magnetic field was also carried out in order to get rid off washing solution that was applied several times during each round of panning to resuspend the cells and thus reduce the phage background. Specifically bound phage particles were finally eluted from the cells by HCl-Glycine pH 2.2 and after neutralization with 2 M Tris pH 12, the eluate was used for infection of a new uninfected *E. coli* XL1 Blue culture. Cells successfully transduced with a pComb3H phagmid copy, encoding a murine Fab-fragment, were again selected for carbenicilline resistance and subsequently infected with VCMS13 helper phage to start another round of antibody display and in vitro selection.

The complete in vitro selection procedure consisted of two initial rounds of panning on immobilized zeta-peptide-BSA-conjugate followed by one round of panning on $CD8^+$-T-lymphocytes and subsequently on $CD56^+$-NK-cells respectively. Then another two rounds of panning were carried out on immobilized zeta-peptide-BSA-conjugate again followed by one round of panning on $CD8^+$-T-cells and finally on $CD56^+$-NK-cells, respectively. Panning on immobilized antigen was performed as described (Barbas et al, Proc Natl Acad Sci USA 88, 7978-82 (1991) in order to keep the selection pressure on zeta-chain specific Fab-fragments that may otherwise be lost by unspecific elution of phage particles, from cell surfaces containing a big number of different antigens in addition to the zeta chain. After each round of panning, plasmid-DNA was prepared from the resulting E. *coli* culture.

For the production of soluble Fab-proteins the *gene III* DNA fragment was excised from these preparations of plasmid-DNA (*Spe*I/*Nhe*I), thus destroying the translational fusion of the Fab-segment with the *gene III* protein. After religation, this pool of plasmid DNA was transformed into 100 µl heat shock competent E. *coli* XL1 Blue and plated on Carbenicilline LB-Agar. Single colonies were grown in 10 ml LB-Carb-cultures/20 mM $MgCl_2$ and Fab-expression was induced after six hours by adding Isopropyl-β-D-thiogalactosid (IPTG) to a final concentration of 1 mM.

These cells were harvested after 20 hours by centrifugation and through four rounds of freezing at -70°C and thawing at 37°C the outer membrane of the bacteria was destroyed by temperature shock so that the soluble periplasmatic proteins including the Fab antibody-fragments were released into the liquid. After elimination of intact cells and cell-debris by centrifugation, the supernatant was tested by ELISA for Fab-antibody-fragments binding to the zeta-peptide-BSA-conjugate.
Detection of Fab-fragments bound to immobilized zeta-peptide-BSA-conjugate was carried out using a horse radish peroxidase conjugated $F(ab')_2$ fragment of a goat anti-mouse IgG + IgM antibody (0,16µg/ml) (Pierce, Rockford, USA, Prod. No. 311448). The signal was developed by adding a substrate solution, containing 2,2'Azino-bis(3-Ethylbenz-Thiazoline-6-Sulfonic Acid) and Na-perborate and detected at a wavelength of 405 nm.

In contrast to clones taken from the library prior to the in vitro selection, those clones tested after different rounds of panning proved to be positive in the zeta-peptide-ELISA with an overall increasing frequency up to the seventh round of panning, which was carried out with purified $CD8^+$-T-cells (Figure 2). However, from the seventh to the eighth round of panning, the latter of which was performed with purified NK-cells, the number of positive clones dropped significantly. This indicates that clones that could be enriched by virtue of their binding activity on T-cells did mostly not correct with NK-cells with the only exception of clone 90 still present after the final panning step on NK-cells.

To check the binding activity of zeta-peptide-reactive Fab-fragments on $CD8^+$-T-cells and NK-cells a flowcytometric analysis was performed. 1×10^6 PBMC were incubated in 50 µl undiluted periplasma preparation for 30 min. on ice followed by incubation with a fluorescein (FITC) conjugated $F(ab')_2$-fragment of a goat anti-mouse IgG + IgM antibody (Jackson Immunoresearch laboratories, West Grove, USA Code No. 115-096-068) diluted 1:100 in PBS. To avoid unspecific binding during the following labeling steps the free valences on the FITC-antibody were blocked by addition of 50 µl 1:10 diluted mouse serum (Sigma immunochemicals, Deisenhofen, M-5905) for 30 minutes. To distinguish the two PBMC-subsets the previously labeled cells were divided. One half was stained with a 1:100 diluted tricolor conjugated anti-CD8

antibody (Caltac Laboratories; USA, Code No. MHCD0306); the other half was stained with a 1:25 diluted phycoerythrin conjugated anti-CD56 antibody (Becton Dickinson, Heidelberg, Cat. No. 347747). As negative control Fab-antibody periplasma preparations of an irrelevant specificity were included. Unlabeled anti-CD16 and anti-CD6 antibodies were used as positive controls for NK-cell and $CD8^+$-T-lymphocytes, respectively.

Two-color fluorescence analysis was carried out on a FACS scan (Becton Dickinson) by applying a positive gate for $CD8^+$- and $CD56^+$-cells, respectively thus allowing the detection of FITC-mediated fluorescence separately on $CD8^+$-T-lymphocytes and NK-cells. Labeling of cells and fluorescence measurements were performed as described in Current Protocols in Immunology (Coligan, Kruisbeek, Margulies, Shevach and Strober, Wiley-Interscience, 1992).

Using the above mentioned protocol 60 different clones, which proved to react with the zeta-peptide-BSA-conjugate in the ELISA-analysis, were analyzed on PBMC. Despite clear positive FACS-signals exhibited by the positive controls, none of the periplasma preparations of anti-zeta chain Fab-antibody-fragments could be demonstrated to bind on the surface of $CD8^+$-T-lymphocytes of NK-cells. This result may be explained by low affinity interactions of Fab-fragments selected by the panning procedure with the surface of T-cells, which may be sufficient for enrichment during the in vitro selection but below the sensitivity of the flowcytometric analysis. However disappearance of zeta-peptide-reactive clones during the final selection step carried out on NK-cells strongly indicates that these potentially T-cell reactive clones did not bind at all to the surface of NK-cells. On the other hand, clone 90, which first appeared during the final round of panning on NK-cells as determined by comparing its variable region sequences with those of other zeta-peptide-reactive clones that appeared earlier during the in vitro selection, most likely exhibited low affinity binding to the NK-cell surface without interacting with T-cells. Accordingly, clone 90 did not appear prior to the second round of panning on NK-cells and no binding signal was detectable on either T-cells or NK-cells by flowcytometric analysis of the corresponding Fab-antibody fragments.

Example 3: Immunization of rats with zeta-peptide-KLH-conjugate and production of anti-zeta-antibodies by the hybridoma technology

At the age of 3 month a Spargue Dawley rat was immunized with the human T-cell line Jurkat (ATCC TIB-152) by intraperitoneal injection of 1 x 10^7 cells. Three months later the animal was immunized with the zeta-peptide-KLH-conjugate. The conjugate was dissolved in 0.9% NaCl at a concentration of 200 µg/ml. The solution was emulsified 1:2 with complete Freund's adjuvants and 100 µl were injected intraperitonially and subcutaneously. The rat received a booster immunization after 4 weeks in the same way, except that no adjuvants was added. Three days after the boost, the animal was sacrificed and the spleen cells were fused with P3X63Ag8.653 -cells (ATCC CRL-1580) to generate hybridoma cell lines following standard protocols. After PEG-fusion, cells were seeded at 100.000 cells per well in microtiter-plates and were grown in 200 µl RPMI 1640 medium supplemented with 10% fetal bovine serum and HAT-additive for selection. After 8 days culture supernatant was completely removed and replaced by fresh medium. After another 4 days, culture supernatant from each well was diluted 1:1 and tested by ELISA (see Example 1). Supernatants from those 45 wells exhibiting the strongest reactions with immobilized zeta-peptide-BSA-conjugate were selected for FACS-analysis on $CD8^+$-T-lymphocytes and NK-cells that was carried out as described in Example 1 for the murine monoclonal antibodies except that a FITC-labeled anti-rat immunoglobulin antibody (IgG + IgM) (Dianova/Jackson, Hamburg, Cat. No. 112-016-044) was used instead of the FITC-conjugated $F(ab')_2$-fragment of a rabbit anti-mouse Ig antibody. Of the 45 different rat monoclonal antibodies tested, only one clone designated 2-B-5 proved to bind on both $CD8^+$-T-lymphocytes and NK-cells. Therefore this clone was analyzed in more detail as described in Examples 4 – 7.

Example 4: Flowcytometric analysis of the anti-zeta-chain antibody 2-B-5 on $CD8^+$-T-cells and NK-cells

In order to test the binding activity of the 2-B-5 antibody on the surface of $CD8^+$-T-lymphocytes and NK-cells, mononucleated cells from the peripheral blood of two

different healthy donors were isolated by Ficoll-density gradient centrifugation. In each well of a microtiterplate 100.000 mononucleated cells were incubated with undiluted cell culture supernatant of the 2-B-5 hybridoma and with several dilutions thereof, respectively. As negative control culture supernatant of an antibody with the same isotype (rat IgM) but irrelevant specificity was used. After 30 minutes of incubation on ice cells were washed two times with PBS and subsequently stained with two different antibody labeling mixtures. The $CD8^+$-T-cells were simultaneously incubated for half an hour on ice with a fluorescein (FITC) conjugated goat-anti-rat Ig (IgG + IgM) antibody (Dianova/Jackson, Hamburg, Cat. No. 112-016-044) diluted 1:100 in PBS, a phycoerythrin (PE) conjugated CD56 antibody (Becton Dickinson, Heidelberg, Cat. No. 347747) diluted 1:25 in PBS and a tricolor conjugated CD3 antibody (Caltac Laboratories, Burlingame, USA, Cod. No. MHCD0306) diluted 1:50 in PBS. To this labeling mixture mouse serum (Sigma Aldrich, St Louis, USA, Cat. No. 054H-8958) was added at a dilution of 1:10 to avoid unspecific binding reactions of the anti-rat antibody to the mouse antibodies.

The NK-cell fraction was incubated with the same goat-anti-rat Ig antibody diluted 1:100 in PBS, a tricolor conjugated CD8 antibody (Caltac Laboratories, Cod. No. MHCD0806) diluted 1:100 in PBS and a phycoerythrin (PE) conjugated CD16 antibody (Becton Dickinson, Heidelberg, Cat. No. 347617) diluted 1:25 in PBS. This mixture was complemented with mouse serum as well. The labeled cells were washed twice in PBS prior to fixation with PBS / 1% paraformaldehyde.

Cells were analyzed by flowcytometry on a FACS-scan (Becton Dickinson, Heidelberg). FACS staining and measuring of the fluorescence intensity were performed as described in Current Protocols in Immunology (Coligan, Kruisbeek, Margulies, Shevach and Strober, Wiley-Interscience, 1992).

Triple color fluorescence analysis was carried out by applying a positive gate for $CD8^+$ (Tricolor) and a negative gate for $CD16^+$ (PE) cells thus allowing the detection of FITC-mediated fluorescence exclusively attributed to $CD8^+$-T-lymohocytes (phenotype: $CD8^+$, $CD16^-$) without any contaminating signals from $CD8^+$-NK-cells. Similarly, triple color fluorescence analysis was carried out by applying a positive gate for $CD56^+$- (PE) and a negative gate for $CD3^+$-cells (tricolor) thus allowing the detection of FITC-mediated fluorescence exclusively attributed to NK-cells

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
c 1368 PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 99/ 04838	09/07/1999	10/07/1998
Applicant		
CONNEX GMBH et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/ 04838

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claim 27 (partially, as far as an in vivo method is concerned) is directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects ot the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/04838

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/13 C12N15/70 C12N15/85 C12N1/21 C12N5/10
C07K16/28 C07K16/46 A61K31/70 A61K39/395 G01N33/577
G01N33/68 C12Q1/68 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M. MACK ET AL.: "Biologic properties of a bispecific single-chain antibody directed against 17-1A (EpCAM) and CD3: tumor cell-dependent T cell stimulation and cytotoxic activity." THE JOURNAL OF IMMUNOLOGY, vol. 158, no. 8, 15 April 1997 (1997-04-15), pages 3965-3970, XP002100040 Baltimore, MD, USA cited in the application the whole document --- -/--	1-29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 November 1999	12/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	A. TRAUNECKER ET AL.: "Bispecific single chain molecules (Janusins) target cytotoxic lymphocytes on HIV infected cells." THE EMBO JOURNAL, vol. 10, no. 12, December 1991 (1991-12), pages 3655-3659, XP000232579 Oxford, GB abstract figure 1 ---	1-29
A	W. HELFRICH ET AL.: "Construction and characterization of a bispecific diabody for retargeting T cells to human carcinomas." INTERNATIONAL JOURNAL OF CANCER, vol. 76, no. 2, 13 April 1998 (1998-04-13), pages 232-239, XP002121156 Copenhagen, Denmark abstract material and methods figure 4 ---	1-29
A	C. RENNER ET AL.: "T cells from patients with Hodgkin's disease have a defective T-cell receptor zeta chain expression that is reversible by T-cell stimulation with CD3 and CD28." BLOOD, vol. 88, no. 1, 1 July 1996 (1996-07-01), pages 236-241, XP002121157 New York, NY, USA abstract page 237, left-hand column, line 1 - line 14 ---	1-29
A	J. SMITH ET AL.: "Nonmitogenic anti-CD3 monoclonal antibodies deliver a partial T cell recetor signal and induce clonal anergy." THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 185, no. 8, 21 April 1997 (1997-04-21), pages 1413-1422, XP002121158 New York, NY, USA abstract page 1414, right-hand column, line 12 - line 45 ---	1-29
A	WO 90 15822 A (DANA-FARBER CANCER INSTITUTE) 27 December 1990 (1990-12-27) the whole document -----	1-29

1

INTERNATIONAL SEARCH REPORT

nation on patent family members

ternational Application No
CT/EP 99/04838

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9015822 A	27-12-1990	NONE	

PATENT COOPERATION TREA

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
VOSSIUS & PARTNER
Attn. VOSSIUS & PARTNER
Postfach 86 07 67
D-81634 München
GERMANY

EINGEGANGEN
Vossius & Partner
15. Nov. 1999
Frist bearb.: 12.12.xx

Date of mailing *(day/month/year)* 12/11/1999	
Applicant's or agent's file reference: c 1368 PCT	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No.: PCT/EP 99/04838	International filing date *(day/month/year)* 09/07/1999
Applicant: CONNEX GMBH et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Barbara Klaver

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
c 1368 PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 99/ 04838	09/07/1999	10/07/1998
Applicant		
CONNEX GMBH et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/ 04838

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claim 27 (partially, as far as an in vivo method is concerned) is directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects ot the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/04838

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/13 C12N15/70 C12N15/85 C12N1/21 C12N5/10
C07K16/28 C07K16/46 A61K31/70 A61K39/395 G01N33/577
G01N33/68 C12Q1/68 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M. MACK ET AL.: "Biologic properties of a bispecific single-chain antibody directed against 17-1A (EpCAM) and CD3: tumor cell-dependent T cell stimulation and cytotoxic activity." THE JOURNAL OF IMMUNOLOGY, vol. 158, no. 8, 15 April 1997 (1997-04-15), pages 3965-3970, XP002100040 Baltimore, MD, USA cited in the application the whole document --- -/--	1-29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 November 1999	12/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	A. TRAUNECKER ET AL.: "Bispecific single chain molecules (Janusins) target cytotoxic lymphocytes on HIV infected cells." THE EMBO JOURNAL, vol. 10, no. 12, December 1991 (1991-12), pages 3655-3659, XP000232579 Oxford, GB abstract figure 1 ---	1-29
A	W. HELFRICH ET AL.: "Construction and characterization of a bispecific diabody for retargeting T cells to human carcinomas." INTERNATIONAL JOURNAL OF CANCER, vol. 76, no. 2, 13 April 1998 (1998-04-13), pages 232-239, XP002121156 Copenhagen, Denmark abstract material and methods figure 4 ---	1-29
A	C. RENNER ET AL.: "T cells from patients with Hodgkin's disease have a defective T-cell receptor zeta chain expression that is reversible by T-cell stimulation with CD3 and CD28." BLOOD, vol. 88, no. 1, 1 July 1996 (1996-07-01), pages 236-241, XP002121157 New York, NY, USA abstract page 237, left-hand column, line 1 - line 14 ---	1-29
A	J. SMITH ET AL.: "Nonmitogenic anti-CD3 monoclonal antibodies deliver a partial T cell recetor signal and induce clonal anergy." THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 185, no. 8, 21 April 1997 (1997-04-21), pages 1413-1422, XP002121158 New York, NY, USA abstract page 1414, right-hand column, line 12 - line 45 ---	1-29
A	WO 90 15822 A (DANA-FARBER CANCER INSTITUTE) 27 December 1990 (1990-12-27) the whole document -----	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 99/04838

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9015822 A	27-12-1990	NONE	

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

VOSSIUS & PARTNER
VOSSIUS & PARTNER
Siebertstrasse 4
81675 München
ALLEMAGNE

EINGEGANGEN
Vossius & Partner
23. Okt. 2000
Frist
bearb.:

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Applicant's or agent's file reference c 1368 PCT	Date of mailing *(day/month/year)* 18. 10. 00
	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/04838	09/07/1999	10/07/1998

Applicant
CONNEX GMBH et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Vullo, C Tel. +49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
c 1368 PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/04838	09/07/1999	10/07/1998
International Patent Classification (IPC) or national classification and IPC		
C12N15/13		

Applicant

CONNEX GMBH et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☒ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
26/01/2000	18. 10. 00
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Armandola, E Telephone No. +49 89 2399 7493

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP99/048

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-57 as originally filed

Claims, No.:

1-38 as received on 10/08/2000 with letter of 10/08/2000

Drawings, sheets:

1/13-13/13 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 36 (Industrial applicability).

because:

☒ the said international application, or the said claims Nos. 36 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-38
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-38
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-35, 37, 38
	No:	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item I

Basis of the report

The description, pages 1-57, includes pages 1-49 as originally filed and 8 pages of Sequence Listing (pages 50-57).

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Industrial Applicability (Art 33 (4) PCT)

Claim 36 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Art. 34(4)(a)(i) PCT).

For the assessment of the present Claim 36, with regard to a method of diagnosis, on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claim. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: M. MACK ET AL.: 'Biologic properties of a bispecific single-chain antibody directed against 17-1A (EpCAM) and CD3: tumor cell-dependent T cell stimulation and cytotoxic activity.' THE JOURNAL OF IMMUNOLOGY, vol. 158, no. 8, 15 April 1997

(1997-04-15), pages 3965-3970, XP002100040 Baltimore, MD, USA cited in the application

D2: W. HELFRICH ET AL.: 'Construction and characterization of a bispecific diabody for retargeting T cells to human carcinomas.' INTERNATIONAL JOURNAL OF CANCER, vol. 76, no. 2, 13 April 1998 (1998-04-13), pages 232-239, XP002121156 Copenhagen, Denmark

D1 discloses the production of a single chain bispecific antibody directed to CD3 and EpCAM to be used to redirect cytotoxic T cells to tumor cells in an MHC-unrestricted fashion.

D2 discloses the construction of a recombinant bispecific antibody fragment in the diabody format, specific for both the CD3 ε-chain and for the tumor antigen EGP2. This diabody is useful to retarget activated cytotoxic T cell to lyse various human carcinoma in vivo.

Novelty and Inventive step (Art. 33 (2)(3) PCT)

Claims 1-38 can be considered novel and inventive for the following reasons:
an antibody recognizing the extracellular portion of the TCR-complex-linked zeta-chain, whose CDRs are encoded by the nucleic acid sequences of SEQ. ID. NO: 1, 3, 5, 7, 9, 11 and whose V_H- and V_L-chain are encoded by the nucleic acids of SEQ. ID. NO: 13 and 15, respectively, was not known in the prior art. Also the application of such an antibody and of the nucleic acid encoding it, such as the production of a bispecific antibody, the preparation of a pharmaceutical composition, the development of a method to determine zeta- or eta-chain expression on the cell surface or a transgenic animal expressing the antibody or one of its derivatives, have not been previously disclosed.

The production of such antibody is no trivial task, in view of the length of the extracellular portion of the zeta-chain (only 9 amino acids) and of the high degree of conservation of its sequence among species (100% identity in mouse, rat and human) which make it difficult to raise antibodies recognizing it on the surface of cells.
The skilled person aiming at producing such an antibody and determining the corresponding nucleotide sequences, would have been aware of the problems connected

with its production, but would have found no hints in the available prior art as to how to arrive at the solution offered in the claims of the present application with reasonable expectation of success, without the exercise of inventive skills.

The concept of producing bispecific antibodies to bring cytotoxic T cells and target cells (e.g tumor cells or virally infected cells) into direct contact is not new (see D1 and D2 as examples of a vast body of literature on the subject). However, in view of the structural features of the zeta-chain (i.e. very short extracellular domain), it does not appear obvious to exploit this molecule to redirect cytotoxic T cells to a target.

For the same reasons uses of the antibody and its derivatives, pharmaceutical compositions, kits, methods and transgenic animals comprising it are also novel and inventive.

Re Item VII

Certain defects in the international application

The dependence of Claim 13 might be wrongly indicated: as its subject-matter refers to a V_L chain its seems more likely that the claim should depend on Claim 8 than on Claim 7 as presently indicated.

Re Item VIII

Certain observations on the international application

Clarity (Art. 6 PCT)

Claims 7 and 8 are not clear due to the expression "said antibody". The preamble of the claim contains no reference to an antibody but only to an antibody chain (V_H or V_L).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
c 1368 PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/04838	09/07/1999	10/07/1998
International Patent Classification (IPC) or national classification and IPC C12N15/13		
Applicant CONNEX GMBH et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
26/01/2000	18. 10. 00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Armandola, E Telephone No. +49 89 2399 7493

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP99/04838

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-57	as originally filed				

Claims, No.:

1-38	as received on	10/08/2000	with letter of	10/08/2000	

Drawings, sheets:

1/13-13/13	as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 36 (Industrial applicability).

because:

☒ the said international application, or the said claims Nos. 36 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-38
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-38
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-35, 37, 38
	No:	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item I

Basis of the report

The description, pages 1-57, includes pages 1-49 as originally filed and 8 pages of Sequence Listing (pages 50-57).

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Industrial Applicability (Art 33 (4) PCT)

Claim 36 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Art. 34(4)(a)(i) PCT).

For the assessment of the present Claim 36, with regard to a method of diagnosis, on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claim. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: M. MACK ET AL.: 'Biologic properties of a bispecific single-chain antibody directed against 17-1A (EpCAM) and CD3: tumor cell-dependent T cell stimulation and cytotoxic activity.' THE JOURNAL OF IMMUNOLOGY, vol. 158, no. 8, 15 April 1997

(1997-04-15), pages 3965-3970, XP002100040 Baltimore, MD, USA cited in the application

D2: W. HELFRICH ET AL.: 'Construction and characterization of a bispecific diabody for retargeting T cells to human carcinomas.' INTERNATIONAL JOURNAL OF CANCER, vol. 76, no. 2, 13 April 1998 (1998-04-13), pages 232-239, XP002121156 Copenhagen, Denmark

D1 discloses the production of a single chain bispecific antibody directed to CD3 and EpCAM to be used to redirect cytotoxic T cells to tumor cells in an MHC-unrestricted fashion.

D2 discloses the construction of a recombinant bispecific antibody fragment in the diabody format, specific for both the CD3 ε-chain and for the tumor antigen EGP2. This diabody is useful to retarget activated cytotoxic T cell to lyse various human carcinoma in vivo.

Novelty and Inventive step (Art. 33 (2)(3) PCT)

Claims 1-38 can be considered novel and inventive for the following reasons:
an antibody recognizing the extracellular portion of the TCR-complex-linked zeta-chain, whose CDRs are encoded by the nucleic acid sequences of SEQ. ID. NO: 1, 3, 5, 7, 9, 11 and whose V_H- and V_L-chain are encoded by the nucleic acids of SEQ. ID. NO: 13 and 15, respectively, was not known in the prior art. Also the application of such an antibody and of the nucleic acid encoding it, such as the production of a bispecific antibody, the preparation of a pharmaceutical composition, the development of a method to determine zeta- or eta-chain expression on the cell surface or a transgenic animal expressing the antibody or one of its derivatives, have not been previously disclosed.

The production of such antibody is no trivial task, in view of the length of the extracellular portion of the zeta-chain (only 9 amino acids) and of the high degree of conservation of its sequence among species (100% identity in mouse, rat and human) which make it difficult to raise antibodies recognizing it on the surface of cells.
The skilled person aiming at producing such an antibody and determining the corresponding nucleotide sequences, would have been aware of the problems connected

with its production, but would have found no hints in the available prior art as to how to arrive at the solution offered in the claims of the present application with reasonable expectation of success, without the exercise of inventive skills.

The concept of producing bispecific antibodies to bring cytotoxic T cells and target cells (e.g tumor cells or virally infected cells) into direct contact is not new (see D1 and D2 as examples of a vast body of literature on the subject). However, in view of the structural features of the zeta-chain (i.e. very short extracellular domain), it does not appear obvious to exploit this molecule to redirect cytotoxic T cells to a target.

For the same reasons uses of the antibody and its derivatives, pharmaceutical compositions, kits, methods and transgenic animals comprising it are also novel and inventive.

Re Item VII

Certain defects in the international application

The dependence of Claim 13 might be wrongly indicated: as its subject-matter refers to a V_L chain its seems more likely that the claim should depend on Claim 8 than on Claim 7 as presently indicated.

Re Item VIII

Certain observations on the international application

Clarity (Art. 6 PCT)

Claims 7 and 8 are not clear due to the expression "said antibody". The preamble of the claim contains no reference to an antibody but only to an antibody chain (V_H or V_L).

PCT/EP99/04838
CONNEX GmbH et al.
Our Ref.: C 1368 PCT

CLAIMS

1. A nucleic acid molecule comprising a nucleic acid sequence encoding at least one complementary determining region (CDR) of a variable region of an antibody, wherein said at least one CDR alone or in combination with at least one more CDR being sufficient for contributing at least a weak but significant binding of the antibody to the extracellular domain of the human zeta-chain and said antibody being obtainable by immunizing a rat with Jurkat cells and subsequently with a conjugate comprising a carrier molecule and a peptide comprising the 11 N-terminal amino acids of the rat zeta-chain.

2. The nucleic acid molecule of claim 1 wherein said nucleic acid molecule comprises a nucleic acid sequence encoding at least two CDRs of said variable region.

3. The nucleic acid molecule of claim 1 or 2, wherein said nucleic acid molecule comprises a nucleic acid sequence encoding three CDRs of said variable region.

4. The nucleic acid molecule of any one of claims 1 to 3 wherein said nucleic acid sequence encodes a V_H chain.

5. The nucleic acid molecule of any one of claims 1 to 3 wherein said nucleic acid sequence encodes a V_L chain.

6. The nucleic acid molecule of any one of claims 1 to 5 which is a DNA molecule.

7. A nucleic acid molecule comprising a nucleic acid sequence encoding at least two CDRs of a variable region of a V_H chain, said antibody specifically interacting with the extracellular domain of the human zeta chain said

antibody being obtainable by immunizing a rat with Jurkat cells and subsequently with a conjugate comprising a carrier molecule and a peptide comprising the 11 N-terminal amino acids of the rat zeta-chain.

8. A nucleic acid molecule comprising a nucleic acid sequence encoding at least two CDRs of a variable region of a V_L chain, said antibody specifically interacting with the extracellular domain of the human zeta chain said antibody being obtainable by immunizing a rat with Jurkat cells and subsequently with a conjugate comprising a carrier molecule and a peptide comprising the 11 N-terminal amino acids of the rat zeta-chain.

9. The nucleic acid molecule of any one of claims 1 to 8 wherein said CDR has one of the following nucleotide sequences:
   (a) SEQ ID No. 1
   (b) SEQ ID No. 3
   (c) SEQ ID No. 5
   (d) SEQ ID No. 7
   (e) SEQ ID No. 9
   (f) SEQ ID No. 11

10. The nucleic acid molecule of claim 4 wherein said V_H-chain has the nucleotide sequence of SEQ ID No. 13 or encodes the amino acid sequence of SEQ ID No. 14.

11. The nucleic acid molecule of claim 5 wherein said V_L-chain has the nucleotide sequence of SEQ ID No. 15 or encodes the amino acid sequence of SEQ ID No. 16.

12. The nucleic acid molecule of claim 7, wherein said V_H chain has the nucleotide sequence of SEQ ID NO:13 or encodes the amino acid sequence of SEQ ID NO:14.

13. The nucleic acid molecule of claim 7, wherein said V_L chain has the nucleotide sequence of SEQ ID NO:15 or encodes the amino acid sequence of SEQ ID NO:16.

14. The nucleic acid molecule of any one of claims 1 to 8 wherein the CDR encodes one of the amino acid sequences:
(a) SEQ ID No. 2
(b) SEQ ID No. 4
(c) SEQ ID No. 6
(d) SEQ ID No. 8
(e) SEQ ID No. 10
(f) SEQ ID No. 12

15. A vector comprising the nucleic acid molecule of any one of claims 1 to 14.

16. A host transformed or transfected with the vector of claim 15.

17. A method of producing a (poly)peptide encoded by the nucleic acid molecule of any one of claims 1 to 14 comprising culturing the host of claim 16 under suitable conditions and isolating said (poly)peptide from the culture.

18. A (poly)peptide encoded by the nucleic acid molecule of any of claims 1 to 14 or produced by the method of claim 17.

19. An antibody or fragment or derivative thereof comprising at least one (poly)peptide of claim 18.

20. The antibody of claim 19 which is a monoclonal antibody.

21. The antibody of claim 19 which is a bispecific antibody.

22. The antibody of claim 21 wherein the first specificity is for the extracellular domain of the human zeta-chain on the surface of an intact cell and the

second specificity is for an optionally different molecule on the surface of a T-lymphocyte, a natural killer cell or a precursor thereof.

23. The antibody of claim 21 wherein the first specificity is for the extracellular domain of the human zeta-chain on the surface of an intact cell and the second specificity is for a different molecule on the surface of a different cell.

24. The antibody of claim 23, wherein said different cell is a cell different from a T-cell, an NK-cell or a precursor thereof.

25. The antibody of claim 23 or 24, wherein said different molecule is a virus encoded antigene, a tumor associated antigen or a surface antigen either on antigen presenting cells (APCs) or on non-APCs.

26. The antibody of claim 25, wherein the APC is a dendritic cell.

27. The derivative of claim 19 which is an scFv chain.

28. The antibody of claim 20 which is an IgM.

29. A bispecific receptor comprising a (poly)peptide of claim 18 and a natural receptor, natural ligand or derivatives thereof interacting with a surface molecule on the same or on another cell.

30. The bispecific receptor of claim 29, wherein said receptors or ligands are CD4, CTLA-4, B7-1, B7-2, LFA-3, ICAM-1, -2, -3 or chemokines like MIP-1α, MIP-1β, RANTES or SDF-1.

31. A pharmaceutical composition comprising the nucleic acid molecule of any of claims 1 to 14, the vector of claim 15, the host of claim 16, the (poly)peptide of claim 18, the antibody or fragment or derivative thereof of any one of claims 19 to 28 and/or the bispecific receptor of claim 29 or 30.

32. Use of the antibody of claim 22 for the preparation of a pharmaceutical composition for the treatment or prevention of autoimmune diseases, immune deficiencies, T-cell malignancies, infectious diseases or for the suppression of immune response.

33. The use of claim 32, wherein said suppression of immune response is to be in order to avoid graft rejection after organ transplantation.

34. Use of the antibody claim 23 for the preparation of a pharmaceutical composition of the treatment or prevention of malignancies, viral infections, or other infectious diseases.

35. Use of the (poly)peptide of claim 18 or the antibody or fragment or derivative thereof of any one of claims 19 to 28 or the bispecific receptor of claim 29 or 30 for the preparation of a pharmaceutical composition for the enhancement or suppression of NK-cell dependent immunity or for the treatment of NK-cell derived malignancies.

36. A method for the determination of zeta-chain or eta-chain expression on NK-cells, T-lymphocytes or precursors thereof comprising
(a) contacting the (poly)peptide of claim 18 or the antibody or fragment or derivative thereof of any one claims 19 to 28 with said NK-cells, T-lymphocytes or precursors thereof; and
(b) assessing the amount of bound (poly)peptide, antibody or derivative.

37. A kit comprising the nucleic acid molecule of any of claims 1 to 14, the vector of claim 15, the host of claim 14, the (poly)peptide of claim 18, the antibody or fragment or derivative thereof of any one of claims 19 to 28 and/or the bispecific receptor of claim 29 or 30.

38. A non-human transgenic animal comprising in its germline at least one copy of the nucleic acid molecule of any of claims 1 to 14 or the vector of claim 15.

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

VOSSIUS & PARTNER
VOSSIUS & PARTNER
Siebertstrasse 4
D-81675 München
ALLEMAGNE

EINGEGANGEN
Vossius & Partner
20. April 2000
Frist
bearb.:

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	19.04.2000
Applicant's or agent's file reference c 1368 PCT	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/04838	09/07/1999	10/07/1998

International Patent Classification (IPC) or both national classification and IPC

C12N15/13

Applicant

CONNEX GMBH et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 10/11/2000.

Name and mailing address of the international preliminary examining authority:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner

Armandola, E

Formalities officer (incl. extension of time limits)
Vullo, C
Telephone No. +49 89 2399 8061

Form PCT/IPEA/408 (cover sheet) (January 1994)

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-57 as originally filed

Claims, No.:

1-29 as originally filed

Drawings, sheets:

1/13-13/13 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application,

☒ claims Nos. (1-6) complete and (11-14, 23, 28, 29) partially (N, IS); 27 (IA) ,

because:

☒ the said international application, or the said claims Nos. 27 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 1-6, 11-14, 23; 28, 29 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims
Inventive step (IS)	Claims
Industrial applicability (IA)	Claims

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item I
Basis of the opinion

The description, pages 1-57, includes pages 1-49 as originally filed and 8 pages of Sequence Listing (pages 50-57).

Re Item III
Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Novelty and Inventive step (Art. 33(2)(3) PCT)

Claims 1-6 were not examined for novelty and inventive step, due to the insufficient characterization of their subject-matter. The same applies to the part(s) of Claims 11-14, 23, 28 and 29 dependent on Claims 1-6. For additional comments, see Item VIII.

Industrial Applicability (Art 33 (4) PCT)

Claim 27 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Art. 34(4)(a)(i) PCT).

For the assessment of the present Claim 27, with regard to a method of diagnosis, on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claim. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item V
Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: M. MACK ET AL.: 'Biologic properties of a bispecific single-chain antibody directed against 17-1A (EpCAM) and CD3: tumor cell-dependent T cell stimulation and cytotoxic activity.' THE JOURNAL OF IMMUNOLOGY, vol. 158, no. 8, 15 April 1997 (1997-04-15), pages 3965-3970, XP002100040 Baltimore, MD, USA cited in the application

D2: W. HELFRICH ET AL.: 'Construction and characterization of a bispecific diabody for retargeting T cells to human carcinomas.' INTERNATIONAL JOURNAL OF CANCER, vol. 76, no. 2, 13 April 1998 (1998-04-13), pages 232-239, XP002121156 Copenhagen, Denmark

D1 discloses the production of a single chain bispecific antibody directed to CD3 and EpCAM to be used to redirect cytotoxic T cells to tumor cells in an MHC-unrestricted fashion.

D2 discloses the construction of a recombinant bispecific antibody fragment in the diabody format, specific for both the CD3 ε-chain and for the tumor antigen EGP2. This diabody is useful to retarget activated cytotoxic T cell to lyse various human carcinoma in vivo.

Novelty and Inventive step (Art. 33 (2)(3) PCT)

Claims 7-10, 15-22, 24-27 and those parts of Claims 11-14, 23, 28 and 29 not referring to Claims 1-6 may be considered novel and inventive:
an antibody recognizing the extracellular portion of the TCR-complex-linked zeta-chain, whose CDRs are encoded by the nucleic acid sequences of SEQ. ID. NO: 1, 3, 5, 7, 9, 11 and whose VH- and VL-chain are encoded by the nucleic acids of SEQ. ID. NO: 13 and 15, respectively, was not known in the prior art. Also the application of such an antibody and of the nucleic acid encoding it, such as the production of a bispecific antibody, the preparation of a pharmaceutical composition, the development of a method to determine zeta- or eta-chain expression on the cell surface or a transgenic animal expressing the antibody or one of its derivatives, have not been previously disclosed.
The skilled person aiming at producing such an antibody and determining the corresponding nucleotide sequences, would have been aware of the problems connected with its production, but would have found no hints in the available prior art (e.g. D1 and D2) as to how to arrive at the solution offered in the claims of the present application with

reasonable expectation of success, without the exercise of inventive skills.

Re Item VIII
Certain observations on the international application

Clarity (Art. 6 PCT)

i) Claim 1 is unclear because its subject-matter is not sufficiently characterized. Structurally and functionally different antibodies reacting with the extracellular portion of the human zeta-chain of the TCR complex may be obtained by the immunization protocol described in the claim. Such antibodies may be constituted by any combination between heavy and light chains which, in turn, are coded for by nucleic acids whose sequence is the result of recombination and mutation events. It would, therefore, be impossible for the skilled person to precisely identify the nucleic acid(s) for which protection is sought among all the possible molecules which might fulfill the requirements of the claim, without a more precise characterization through structural features, such as their nucleic acid sequence. In addition, while antibody specificity might be determined by one CDR only, it is often the combination of specific heavy and light chains and of two or more CDRs on them, which determines the recognition pattern of an antibody. Antibodies may share one or more CDRs, and still have a different specificities. Therefore, the subject-matter of Claim 1 may not be novel as it encompasses nucleic acid molecules encoding known antibodies, which could comprise at least one CDR of a variable region of an antibody interacting with the extracellular domain of the human zeta-chain, obtained by the method specified in the claim, without having a specificity related to the human zeta-chain. For example, a nucleic acid sequence encoding an antibody (e.g. a single chain antibody) with the same light chain of the antibody described in the claim but with a greatly different heavy chain which determines its antigenic specificity would also fall within the scope of the claim.

In view of the objection above also Claims 2-6 and the part(s) of Claims 11-14, 23, 29 and 29 referring to Claim 1-6 are unclear.

ii) In Claims 15 and 22 the terms "fragment" and "derivative", without any structural or functional limitation, are considered to be vague and unclear as they leave the reader in doubt as to the meaning of the technical features to which they refer, thereby rendering the scope of the claims unclear and open to interpretation.

iii) The terms "preferably" and "most preferably" found in Claims 19, 22 and 24 are not seen as having a limiting effect on the scope of the claim and the features following these terms are regarded as entirely optional (see also PCT Guidelines III-4.6).

iv) The use of the term "animal" in Claim 29, does not exclude that the scope of the claim may extend to genetically transformed human beings. A claim directed to human beings is regarded as being contrary to morality according to Rule 9.1(i) PCT.

The applicant is requested to file new claims which take account of the above comments

The applicant is requested to file amendments by way of replacement pages in the manner stipulated by Rule 66.8(a) PCT. In particular, fair copies of the amendments should be filed preferably in triplicate.

In order to facilitate the examination of the conformity of the amended application with the requirements of Article 34(2)(b) PCT, the applicant is requested to clearly identify the amendments carried out, no matter whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based (see also Rule 66.8(a) PCT).

If the applicant regards it as appropriate these indications could be submitted in handwritten form on a copy of the relevant parts of the application as filed.

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The name or two-letter code of that Authority mat, be indicated by the applicant oil the line below:

IPEA/EP _____

PCT

DEMAND

CHAPTER II

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: C 1368 PCT

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/EP99/04838	09 July 1999 (09/07/99)	10 July 1998 (10/07/98)

Title of invention
Immunological reagent specifically interacting with the extracellular domain of the human zeta chain

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name, for a legal entity: full official designation. The address must include postal code and name of country.)*

CONNEX GMBH
Am Klopferspitz 19
82152 Martinsried
DE

Telephone No.:

Facsimile No.:

Teleprinter No.:

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DE	DE

Name and address: *(Family name followed by given name, for a legal entity; full official designation. The address must include postal code and name of country.)*

REITER, Christian
Rathausstr. 8
85757 Karlsfeld
DE

State (that is, country) of nationality:	State *(that is, country)* of residence:
DE	DE

Name and address: *(Family name followed by given name, for a legal entity; full official designation. The address must include postal code and name of country.)*

State (that is, country) of nationality:	State *(that is, country)* of residence:

☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/EP99/04838

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name, for a legal entity: full official designation. The address must include postal code and name of country.)*

Vossius & Partner
Siebertstrasse 4
81675 Munich

Telephone No.: 089 / 41 30 40

Facsimile No.: 089 / 41 304-111

Teleprinter No.:

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination **to start on the basis of:**

☒ the international application as originally filed

the description ☒ as originally filed
☐ as amended under Article 34

the claims ☒ as originally filed
☐ as amended under Article 19 (together with any accompanying statement)
☐ as amended under Article 34

the drawings ☒ as originally filed
☐ as amended under Article 34

2. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. ☐ The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This checkbox may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments *of* the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: **English**

☒ which is the language in which the international application was filed.
☐ which is the language of a translation furnished for the purposes of international search.
☐ which is the language of publication of the international application.
☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**

International application No.
PCT/EP99/04838

Box No. III CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ nucleotide and or amino acid sequence listing in computer readable form
6. ☐ other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

Munich, January 26, 2000

Dr. Joachim Wachenfeld
European Patent Attorney

Wa/ses

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* C 1368 PCT

Box No. I TITLE OF INVENTION

Immunological reagent specifically interacting with the extracellular domain of the human zeta chain.

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: DE

State *(that is, country)* of residence: DE

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

REITER, Christian
Rathausstr. 8
85757 Karlsfeld
DE

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: DE

State *(that is, country)* of residence: DE

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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81634 Munich
DE

Telephone No. 089-413 040

Facsimile No. 089-413 04 111

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No.V DESIGNAT[redacted]OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ **AP** **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ **EA** **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ **AE** United Arab Emirates
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- ☒ **AU** Australia .....
- ☒ **AZ** Azerbaijan
- ☒ **BA** Bosnia and Herzegovina .....
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- ☒ **BG** Bulgaria .....
- ☒ **BR** Brazil .....
- ☒ **BY** Belarus .....
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- ☒ **ES** Spain .....
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- ☒ **GM** Gambia
- ☒ **HR** Croatia .....
- ☒ **HU** Hungary .....
- ☒ **ID** Indonesia
- ☒ **IL** Israel .....
- ☒ **IN** India .....
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- ☒ **KG** Kyrgyzstan .....
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- ☒ **KR** Republic of Korea .....
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- ☒ **LK** Sri Lanka
- ☒ **LR** Liberia
- ☒ **LS** Lesotho .....
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- ☒ **LU** Luxembourg
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- ☒ **MK** The former Yugoslav Republic of Macedonia .....
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- ☒ **ZA** South Africa .....
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Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

- ☐ .....
- ☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY [redacted]IM ☐ Further pr[redacted]claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 10 July 1998 10/07/98	98112867.1		EP	
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): 1

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA /

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	:	3
description (excluding sequence listing part)	:	49
claims	:	4
abstract	:	1
drawings	:	13
sequence listing part of description	:	8
Total number of sheets	:	78

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☒ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: ENGLISH

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Munich, July 9, 1999

Dr. Joachim Wachenfeld
European Patent Attorney

VOSSIUS & PARTNER
PATENTANWÄLTE
SIEBERTSTR. 4
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Wa/OM/mb

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1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid.

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Date of receipt of the record copy by the International Bureau:

(phenotype: $CD56^+$; $CD3^-$) without any contaminating signals from $CD56^+$-T-lymphocytes.

As shown in Figure 3 the 2-B-5 hybridoma antibody specifically binds to the surface of both T-lymphocytes and NK-cells from different donors.

Example 5: Confirmation of zeta chain specificity of the 2-B-5 antibody by Sandwich-ELISA

A sandwich ELISA was carried out in order to confirm the zeta-chain-specificity of the monoclonal antibody 2-B-5.

For this purpose, cell lysate from purified $CD8^+$-T-lymphocytes, that are known to express the zeta-chain, was prepared and incubated with an immobilized antibody that recognizes the intracellular zeta-chain domain. Zeta-chain molecules from the cell lysate, could than be captured by this antibody and subsequently detected by the 2-B-5 antibody raised against the short extracellular portion of the zeta-chain. Isolation of $CD8^+$-lymphcytes was carried out with paramagnetic beads as described in Example 1. The detailed steps were performed according to manufacturers instructions (Dynal, Oslo, Norway). Purified $CD8^+$-cells were lysed by the detergent NP-40 (Sigma, Deisenhofen) in presence of the protease inhibitor Phenylmethansulfonylfluoride (PMSF) (Merck, Darmstadt). For detailed buffer formulation see Sambrook, Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Hobart, NY (1989).

The sandwich-ELISA was carried out as follows:

A zeta-chain specific antibody (Santa Cruz Biotechnology, Cat-No 1124), which recognizes the amino acids 144-163 at the carboxy-terminus of the zeta chain was coated to wells of a 96 U-bottom plate (Nunc, maxisorb) at a concentration of 5 µg/ml. Coating was performed overnight at 4°C, the following blockade was carried out with 3% BSA in PBS for one hour at room temperature. Subsequently the lysate of $CD8^+$-cells was added undiluted and in several dilutions and incubated for one hour. As negative control, the wells were incubated with PBS instead of the cell lysate. In the following step the purified monoclonal antibody 2-B-5 was added at a concentration of

1 µg/ml and incubated for one hour. Bound 2-B-5 antibody was detected with a biotinylated mouse-anti-rat IgM antibody (Zymed, San Francisco, CA, USA; Cat-No 03-9840; working concentration 400 ng/ml) followed by an Avidin-peroxidase-conjugate (Dako, Hamburg; Code-No P 03347; working concentration 1 µg/ml). The ELISA was finally developed by addition of ABTS-substrate solution (Boehringer Mannheim, Mannheim, Cat-No. 1682008). The colored precipitate was measured at 405 nm using an ELISA-reader.

The rat IgM antibody 2-B-5 was purified from the hybridoma culture supernatant by ion exchange chromatography using a Bakerbond Abx column (J.T. Baker, Greisheim, Germany) according to the manufacturer's manual.

As shown in Figure 4 the monoclonal antibody 2-B-5 binds to zeta-chain molecules from the T-cell lysate captured by an immobilized antibody recognizing the intracellular zeta-chain domain, with the zeta-specific ELISA-signal strictly depending on the lysate dilution and distinctly ranging above that of the negative control.

Example 6: Cloning of the variable regions of zeta antibody 2-B-5 and expression of the corresponding Fab-fragment in *E. coli*

RNA was isolated from 5 x 10^6 cells of the rat hybridoma cell line 2-B-5 according to the method described by Chomczynski et al. (Anal Biochem, vol. 162, p 156-9 1989). The total RNA was reverse transcribed with the MMLV reverse transcriptase Superscript II (Gibco BRL, Eggenstein) according to standard protocols (Sambrook, Cold Spring Harbour Laboratory Press 1989, second edition). Specific priming of cDNA was carried out with two oligonucleotides designated ratcmuRT (GTGCAGGGCCAGAGAAGGCATC) matching with a short sequence of the constant region of the rat µ-chain and ratckRT (GTAGGTCGCTTGTGGGGAAGTCTC) complementary to a part of the 3´-untranslated region of the light (kappa) chain, with each primer being located approximately 70 basepairs (bp) downstream from the end of the nucleotide sequence encoding the transcript IgM-CH1-heavy chain domain or the constant region of the kappa light chain, respectively. In both cases the

nucleotide information was received from the Genebank database (http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/) for the kappa chain: Shepard and Gutman, Accession No. J02574 and for the mu chain: Parker, K.E., Accession No. X68312. The first strand of cDNA was then poly-G tailed using terminal transferase (Pharmacia, Freiburg) according to standard protocol. The tailed cDNA was PCR-amplified using a sense primer containing a poly-C stretch, based on the anchor primer sequence published by Gilliland, L. K. et al., (Tissue Antigens 47, 1-20, 1996) and designated 5´-AncTail (CGTCGATGAGCTCTAGAATTCCCCCCCCCCCCD). This anchor primer was combined with an antisense primer, specific for the nucleotide sequence encoding the C-terminus of the kappa light chain constant region or that of the IgM-CH1 heavy chain domain, respectively. The primers were designated 3´ratck (GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA) and 3´ratcmu (ATTGGGACTAGTCTCAACGACAGCTGGAAT). The PCR was carried out as follows: Primary denaturation: 94°C for 4 min.; 30 cycles of amplification: 93°C for 30 sec.; 55°C for 30 sec.; 72°C for 30 sec.; terminal elongation: 72°C for 3 min. Each of these primers contains a restriction enzyme cleavage site (5´-AncTail: *EcoR*I; 3´ratck: *Xba*I; 3´ratcmu: *Spe*I) which allows cloning of the corresponding PCR-fragments into a plasmid vector digested with *EcoR*I/*Xba*I or *EcoR*I/*Spe*I, respectively; for this purpose the bluescript KS+ plasmid vector (Genebank Accession No X52327) was used, since it also allows easy sequence analysis of the resulting inserts by using common sequencing primers. Due to an internal *Spe*I cleavage site within the variable region of the heavy chain (VH) partial digestion followed by cloning of the full length fragment was necessary to obtain the complete sequence information of the VH-domain. Partial digestion was carried out according to standard protocols (Sambrook, Cold Spring Harbour Laboratory Press 1989, second edition). Several clones of heavy and light chain fragments proved to have identical sequences, respectively, and could be identified to encode either functional VL- or VH-regions (see Figure 6 and 7).

The mature N-terminus of both variable chains was identified by comparing their sequences with those found in Genebank database (http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/) and subsequently a second set of PCR-primers was designed to introduce appropriate restriction enzyme cleavage sites in frame with the coding sequences of the 2-B-5 Fab-antibody fragment and with regard to the requirements for subcloning into a bacterial expression vector. The two

primers were designated 5´RVHZXhoI (CAGGTACAGCTGCTCGAGTCTGGGGC-TGAGCTAG) and 5´RVKZSacI (GTAAATGTGAGCTCCAGATGACACAGTCTCCTG) and used in combination with the 3´ratcmu and 3´ratck primers, respectively. After PCR-amplification and digestion with the appropriate restriction enzyme combinations (*XhoI*/*SpeI* for the heavy chain fragment and *SacI*/*XbaI* for the kappa light chain), the resulting DNA-fragments were cloned separately into the correspondingly prepared bluescript plasmid vector and sequenced for confirmation (for sequencing results see Fig. 6 and 7).

For the expression of the 2-B-5-Fab-fragment in the periplasma of E. *coli* the corresponding kappa light chain was excised from Bluescript KS+ by using the restriction enzymes *SacI*/*XbaI* and subcloned into the vector pComb3HHis prepared by digestion with the same enzymes. The resulting plasmid was then digested with the restriction enzymes *XhoI*/*NheI* and used as vector for subcloning of the 2-B-5 heavy chain Fd-fragment (VH+CH1); this DNA-fragment was excised from the corresponding Bluescript KS+-clone with the restriction enzymes *XhoI*/*SpeI*.

pComb3HHis was derived from pComb3H and pComb3, respectively (Barbas et al, Proc Natl Acad Sci USA 88, 7978-82 (1991)) by the following modification: The pComb3H vector was cleaved with *NheI* and a double stranded oligonucleotide with suitable ends was inserted by ligation. The double stranded oligomer was created through annealing of the two 5'-phosphorylated primers His6s (CTAGCCATCACCATCACCATCACA) and His6as (CTAGTGTGATGGTGATGGTGATGG) (at 94°C, 10 min.; 65° C, 30 min.; 52° C 30 min. and 30° C 10 min.). The primer ends were designed in a way that after fusion with the vector the 3' *NheI* restriction site was destroyed whereas the 5' *NheI* cleavage site remained intact. Finally, the insert was sequenced to confirm successful cloning.

Periplasma preparation was carried out by osmotic shock and tested by ELISA for Fab-fragments binding to the zeta-peptide-KLH-conjugate. For this purpose single colonies of E. *coli* XL1 Blue transformed with pComb3HHis containing the heavy chain Fd-fragment and the kappa light chain of 2-B-5 were grown in 10 ml Super Broth-medium supplemented with Carbenicilline and 20 mM $MgCl_2$ and Fab-

expression was induced after six hours by adding Isopropyl-β-D-thiogalactosid (IPTG) to a final concentration of 1 mM. The cells were harvested after 20 hours, redissolved in 1 ml PBS. By four rounds of freezing at -70°C and thawing at 37°C, the outer membrane of the bacteria was destroyed and the soluble periplasmatic proteins including the Fab-fragments were released into the supernatant. After elimination of intact cells and cell-debris by centrifugation, the supernatant containing the zeta-Fab-antibody-fragment was collected and used for further examination.

Binding of the periplasmatically expressed Fab-fragment of the cloned monoclonal antibody 2-B-5 to the zeta-peptide-KLH-conjugate was analyzed by the following ELISA: The antigen was immobilized on 96 U well ELISA plates (nunc maxisorb) at a concentration of 200 µg/ml in 50 µl phosphate buffered saline (PBS) per well. Coating was carried out at 4°C for 12 hours followed by single washing step with PBS/0,05% Tween. The ELISA was subsequently blocked for 1 hour with PBS/3% bovine serum albumin (BSA) and washed again once. Then Fab-containing periplasma preparations were added undiluted and in several dilutions and incubated for 2 hours. For detection of Fab-fragments bound to the zeta-peptide-KLH-conjugate a murine anti-His-tag antibody (Dianova, Hamburg, cat. no DIA900) diluted 1:200 was used followed by a peroxidase conjugated polyclonal goat anti-mouse IgG (Fc-gamma specific) (Dianova/Jackson, Hamburg, cat no 115-035-071) antibody diluted 1:5000. The ELISA was finally developed by addition of ABTS-substrate solution (Boehringer Mannheim, Mannheim, Cat-No. 1682008). The turnover of colored substrate was measured by an ELISA-Reader at OD 405 nm; the results are shown in Figure 5. As negative controls, wells were incubated with PBS instead of periplasma preparations.

Specific binding of several clones to the zeta-peptide-KLH-conjugate could be detected with the results of clone 8 and 9 shown in Figure 5. Signal intensities distinctly ranged above those of the negative controls and could be titrated with the sample dilutions. Thus zeta-chain specificity of the cloned 2-B-5-Fab-fragment was confirmed.

Example 7: Stimulation of T-Lymphocytes and NK-cells by the anti-zeta-chain antibody 2-B-5

The aim of this experiment was to analyze stimulation and proliferation of T-cells, NK-cells and PBMC induced by the anti-zeta-chain antibody 2-B-5. For this purpose a colorimetric immunoassay based on the measurement of Bromodeoxyuridine (BrdU) incorporation during DNA-synthesis was used (Boehringer Mannheim, Mannheim, Cat. No. 1647229).

The first step of this assay was to coat a 96-well flat-bottom microtiterplate with purified of the 2-B-5 antibody in several dilutions (for purification of 2-B-5 see Example 5). Coating was performed overnight at 4°C. After three times of washing with PBS 100.000 $CD8^+$-T-lymphocytes, NK-cells and unseparated PBMC, respectively, were added in triplicates to the wells of the microtiterplate, $CD8^+$-T-lymphocytes and NK-cells were separated according to the instructions given in Example 2 by using magnetic beads and the primary antibodies anti-CD8 (MT-811) and anti-CD16 (3G8, mouse IgG1, Dianova, Hamburg, Cat. No 0813), respectively.

To control the specificity of 2-B-5 mediated stimulation an antibody of the same isotype (rat IgM) with an irrelevant specificity was used at the same concentrations. The antibody OKT3 (Isotype IgG2a, Ortho., Prod. Code 710320, Johnson + Johnson, New York, USA), which recognizes the human CD3-complex was applied as specific positive control at a coating concentration of 1µg/ml for the stimulation of T-cells and unseparated PBMC, respectively. A murine IgG2a-antibody of irrelevant specificity was used as isotype control for OKT3. A blank control (wells without cells) and a background control (wells without BrdU) were also included. After an incubation period of three days the BrdU-labeling solution was added for 24 hours. During this labeling period, the pyrimidine analogue BrdU is incorporated in place of thymidine into the DNA of proliferating cells. After removing the culture medium the cells were fixed and the DNA was denatured in one step by adding a denaturation solution. The denaturation of the DNA is necessary to improve the accessibility of the incorporated BrdU for detection by the anti-BrdU-antibody, which is conjugated with peroxidase. This antibody binds to the BrdU incorporated in newly synthesized, cellular DNA.

Bound anti-BrdU-antibody was detected by the subsequent substrate reaction. The reaction product was quantified by an ELISA reader. The turnover of colored substrate as measured by the absorbance values at a wavelength of 450 nm directly correlates with the level of DNA-synthesis and thus with the number of proliferating cells. All steps were performed as described in the manual of the kit manufacturer.

The results of this assay as shown in Figure 8 clearly demonstrate, that the antibody 2-B-5 not only binds to the short extracellular region of the zeta-chain on both T-lymphocytes and NK-cells but that it also induces strong stimulation of both cell types by this interaction.

Example 8: Flowcytometric analysis of TCR/CD3 complex internalization induced by the binding of the anti-zeta-chain antibody

For many receptors, activation by ligand binding is rapidly followed by receptor internalization. Accordingly, rapid internalization of the TCR-complex on T-cells is typically observed after binding of anti-CD3-antibodies. Thus, internalization of the 2-B-5 antibody after binding to the TCR-complex via its extracellular zeta-chain epitope would confirm the peculiar specificity of the antibody of the invention. (Boyer, C., Auphan, N., Luton, F., Malburet, J. M., Barad, M., Bizozzero, J. P., Reggio, H., and Schmitt-Verhulst, A. M. (1991). T cell receptor/CD3 complex internalization following activation of a cytolytic T cell clone: evidence for a protein kinase C-independent staurosporine-sensitive step. European Journal Of Immunology 21, 1623-34.)

In order to test receptor internalization after binding of the 2-B-5 antibody to the surface of T-cells, a flowcytometric assay was performed at different temperatures, allowing the disappearance of the surface- bound anti-zeta-chain antibody to be observed.

For this purpose mononucleated cells from the peripheral blood of a healthy donor were isolated by Ficoll-density gradient centrifugation. In each well of a microtiterplate 200.000 mononucleated cells were incubated with the anti-zeta-chain antibody 2-B-5

at a concentration of 1 µg/ml, at 4°C or 37°C for either 30 or 60 minutes, thus giving enough time for capping to occur. As positive control a parallel experiment was carried out with a rat-anti-human CD3 antibody (rat IgG2B)(clone 26-II 6-5). The capping process was terminated by washing twice with cold PBS. To label the cell surface bound antibody, the cells were incubated with a fluorescein (FITC) conjugated goat-anti-rat Ig (IgG + IgM) antibody (Dianova/Jackson, Hamburg, Cat. No. 112-016-044) diluted 1:100 in PBS. As negative control, only the secondary antibody was used. The labeled cells were washed twice in PBS prior to fixation with PBS / 0.1% paraformaldehyde.

Cells were analyzed by flowcytometry on a FACS-scan (Becton Dickinson, Heidelberg). FACS staining and measuring of the fluorescence intensity were performed as described in Current Protocols in Immunology (Coligan, Kruisbeek, Margulies, Shevach and Strober, Wiley-Interscience, 1992).

As the results clearly demonstrated, an apparent shift of fluorescence intensity between the cell samples incubated at 37°C and those incubated at 4°C could be observed after binding of the anti zeta chain antibody 2B5. A similar internalization pattern could be observed after binding of anti-CD3 antibody. In contrast to the receptor internalization at 37°C the samples incubated at 4°C, which is a nonpermissive temperature for capping events, revealed an unaltered fluorescence pattern.

Example 9: Construction of bispecific antibody based on the anti-zeta chain specificity of the invention

To obtain an anti-zeta scFv-fragment, the corresponding VL- and VH-regions cloned into separate plasmid vectors served as templates for a VL- and VH-specific PCR using the oligonucleotide primer pairs 5´VL2B5BsrGI-EcoRV/3´VL2B5GS15 and 5´VH2B5GS15/3´VH2B5BspEI, respectively. Thereby, overlapping complementary sequences were introduced into the PCR-products, that combine to form the coding sequence of a 15-amino acid (Gly4Ser1)3-linker during the subsequent fusion-PCR.

This amplification step was performed with the primer pair 5´VL2B5BsrGI-EcoRV/3´VH2B5BspEI and the resulting fusion product (or rather anti-zeta-chain scFv-fragment) was cleaved with the restriction enzymes EcoRV and BspEI and thus cloned into a plasmid (described in PCT/EP98/07313) a prepared by digestion with the same restriction enzymes containing a scFv-fragment with binding specificity against the EpCAM antigen as well as a histidine tag at the C-terminus for purification and analytic purposes. Subsequently, the DNA-fragment encoding the anti-zeta-chain/anti-EpCAM bispecific single-chain antibody with the domain arrangement $VL_{antiZeta}$-$VH_{antiZeta}$-$VH_{antiEpCAM}$-$VL_{antiEpCAM}$ was subcloned EcoRI/SalI into the mammalian expression vector pEF-DHFR (Mack, M., Riethmüller, G., and Kufer, P. (1995). A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. Proc Natl Acad Sci USA 92, 7021-5).

After sequence confirmation (Fig. 10) the resulting plasmid-DNA was transfected into DHFR-deficient CHO-cells by electroporation; selection for stable transfectants, gene amplification and protein production were performed as described (Mack et al). The bispecific antibody was purified via its C-terminal histidine tag by affinity chromatography on a Ni-NTA-column as described (Mack et al).

List of Primers

5´VL2B5BsrGI/EcoRV	5´-AGG TGT ACA CTC CGA TAT CCA GAT GAC ACA GTC TCC-3´
3´VL 2B5 GS15	5´-GGA GCC GCC GCC GCC AGA ACC ACC ACC ACC TTT CAG CTC CAG CTT GGT CCC-3´
5´VH 2B5 GS15	5´-GGC GGC GGC GGC TCC GGT GGT GGT GGT TCT CAG GTA CAG CTG CAG CAA TCT GG-3´
3´VH 2B5 BspEI	5´AAT CCG GAA GAG ACA GTG ACC AGA GTG-3´

Example 10: Cytotoxic activity of PBMC and CD8$^+$-T-lymphocytes redirected against EpCAM-positive target cells by the bispecific anti-zeta-chain/anti-EpCAM antibody

In this experiment, target cells were labeled with ^{51}Cr, washed, mixed with effector cells at an effector-to-target ratio of 20:1 and subsequently incubated with different concentrations of the bispecific anti-zeta-chain/anti-EpCAM antibody. The amount of ^{51}Cr released into the supernatant through target cell killing was quantitated and the rate of specific lysis calculated for each antibody concentration.

For this assay human peripheral blood mononuclear cells (PBMCs) or cytotoxic T-lymphocytes were isolated as effector cells from a fresh buffy coat of a healthy donor. PBMCs were separated by ficoll density-gradient centrifugation followed by a subsequent centrifugation step (100 g) to remove thrombocytes. In order to isolate cytotoxic T-Lymphocytes a CD8$^+$ Subset column kit (R&D systems, Wiesbaden, Cat-No. HCD8C-1000) was used according to the protocols of the manufacturer. 200 000 unstimulated PBMCs or CD8$^+$ T-lymphocytes were added in a volume of 100 µl of RPMI 1640 medium supplemented with 10% FCS to each well of a round-bottomed microtiter plate, respectively. As target cells, Kato III (ATCC HTB-103), an EpCAM positive gastric cancer cell line, labeled for one hour with Chromium-51 (NEN-Life Science, Köln; Cat-No NEZ030S) (with approximately 100µCi) was used; 10.000 target cells in a volume of 100 µl were added to each well of the microtiter plate. The bispecific antibody was added in concentrations from 40 ng/ml to 5 µg/ml in a volume of 50 µl. The microtiter plates were incubated for 16 h at 37° C, 5 % CO_2. At the end of the incubation period 50 µl supernatant were removed from each well and assayed for released ^{51}Cr in a gamma counter (Wallac, 1480 Wizard 3", Freiburg). Maximal ^{51}Cr release was determined by the lysis of target cells with a buffer containing Triton-X 100 (1.0% in PBS). The spontaneous ^{51}Cr release was determined by incubation of target cells without effector cells and bispecific antibody. Incubation of target cells with bispecific antibody did not result in measurable lysis. Specific lysis was calculated as follows: specific release (%) = [(cpm, experimental release) - (cpm, spontaneous release)] / [(cpm, maximal release) - (cpm, spontaneous release)] x 100. All tests were carried out in triplicates. SD within the triplicates was below 6% in all experiments.

The purity of the isolated $CD8^+$ effector cells was analyzed by flow cytometry, contaminations with NK-cells were excluded by staining with a PE conjugated anti human CD56 antibody. FACS-analysis was performed as described in example 1. The following antibody conjugates were used: FITC anti human CD3; (Pharmingen Cat-No 30104X) 1:50; PE anti human CD56; (Becton Dickinson Cat-No 347747) 1:20; Tricolor mouse anti human CD8 (Caltac Lab Code NoMHCD0806) 1:100 (Becton Dickinson). The results of the FACS analysis confirmed the high purity of the $CD8^+$ cell population (> 99%).

The results (Figure 11) of the Chromium release assay clearly demonstrated the capability of the bispecific anti-zeta-chain/anti-EpCAM antibody to redirect unstimulated PBMCs or $CD8^+$ cytotoxic T-lymphocytes against EpCAM positive KATO III cells. The differences between the specific lysis mediated by unseparated PBMC and isolated cytotoxic T-lymphocytes are explained by the cytotoxic contribution of NK-cells.

Example 11: Cytotoxic activity of NK- cells redirected against EpCAM-positive target cells by the bispecific anti-zeta-chain/anti-EpCAM antibody

This experiment was designed to demonstrate the capability of the bispecific anti-zeta-chain/anti-EpCAM antibody to redirect NK-cells against EpCAM positive target cells. To perform this assay NK-cells were isolated from human peripheral blood mononuclear cells (PBMCs) using an NK-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach; Order No 465-02). The isolation strategy is based on the magnetic depletion of non-NK- cells. T cells, B cells, monocytes, basophils, dendritic cells and platelets are indirectly labeled using a cocktail of hapten-conjugated CD3, CD14, CD19, CD36 and anti-IgE antibodies followed by paramagnetic beads coupled to an anti-hapten monoclonal antibody. Cells associated with magnetic beads were retained by virtue of a magnetic field. The unlabeled NK-cells were washed through the column and remain untouched. 100 000 NK- cells from each of three different healthy donors, were added in a volume of 100 µl RPMI 1640 medium supplemented

with 10% FCS to each well of a round-bottomed microtiter plate, respectively. As target, ^{51}Cr-labeled Kato cells, were added to each well (10 000 target cells per well in a volume of 100 µl each). Bispecific antibody was added in a concentration of 1 µg/ml in a volume of 50 µl. The microtiter plates were incubated for 4h at 37° C, 5 % CO_2. At the end of the incubation period 50 µl supernatant were removed and assayed for released ^{51}Cr in a gamma counter (Wallac, 1480 Wizard 3", Freiburg). Maximal ^{51}Cr release was determined by lysis of target cells with a buffer containing a detergent (1.0% Triton-X 100 in PBS). The spontaneous ^{51}Cr release was determined by incubation of target cells without effector cells and bispecific antibody. Incubation of target cells with bispecific antibody alone did not result in measurable lysis. Specific lysis was calculated as follows: specific release (%)= [(cpm, experimental release) - (cpm, spontaneous release)] / [(cpm, maximal release) - (cpm, spontaneous release)] x 100. All tests were carried out in triplicates. SD within the triplicates was below 6% in all experiments.

The purity of isolated NK-effector cells was analyzed by flow cytometry; contaminations with $CD8^+$ cells were excluded by staining with a Tricolor conjugated mouse anti human CD8 antibody. FACS-analysis was performed as described in example 1. The following antibody conjugates were used: FITC anti human CD3; (Pharmingen Cat-No 30104X) 1:50; PE anti human CD56; (Becton Dickinson Cat-No 347747) 1:20; Tricolor mouse anti human CD8 (Caltac Lab Code NoMHCD0806) 1:100 (Becton Dickinson). The results of the FACS analysis confirmed the high purity of the NK- cell population (> 98%).

The results (Figure 12) of the chromium release assay demonstrated in all 3 cases a reproducible capability of redirecting NK- cells against EpCAM positive target cells.

Claims

1. A nucleic acid molecule comprising a nucleic acid sequence encoding at least one complementary determining region (CDR) of a variable region of an antibody, said antibody specifically interacting with the extracellular domain of the human zeta-chain, said antibody being obtainable by immunizing a rat with Jurkat cells and subsequently with a conjugate comprising a carrier molecule and a peptide comprising the 11 N-terminal amino acids of a the rat zeta-chain.

2. The nucleic acid molecule of claim 1 wherein said nucleic acid molecule comprises a nucleic acid sequence encoding at least two CDRs of said variable region.

3. The nucleic acid molecule of claim 1 or 2, wherein said nucleic acid molecule comprises a nucleic acid sequence encoding three CDRs of said variable region.

4. The nucleic acid molecule of any one of claims 1 to 3 wherein said nucleic acid sequence encodes a V_H chain.

5. The nucleic acid molecule of any one of claims 1 to 3 wherein said nucleic acid sequence encodes a V_L chain.

6. The nucleic acid molecule of any one of claims 1 to 5 which is a DNA molecule.

7. The nucleic acid molecule of any one of claims 1 to 6 wherein said CDR has one of the following nucleotide sequences:
   (a) SEQ ID No. 1
   (b) SEQ ID No. 3
   (c) SEQ ID No. 5
   (d) SEQ ID No. 7
   (e) SEQ ID No. 9

(f) SEQ ID No. 11

8. The nucleic acid molecule of claim 4 wherein said V_H-chain has the nucleotide sequence of SEQ ID No. 13 or encodes the amino acid sequence of SEQ ID No. 14.

9. The nucleic acid molecule of claim 5 wherein said V_L-chain has the nucleotide sequence of SEQ ID No. 15 or encodes the amino acid sequence of SEQ ID No. 16.

10. The nucleic acid molecule of any one of claims 1 to 6 wherein the CDR encodes one of the amino acid sequences:
(a) SEQ ID No. 2
(b) SEQ ID No. 4
(c) SEQ ID No. 6
(d) SEQ ID No. 8
(e) SEQ ID No. 10
(f) SEQ ID No. 12

11. A vector comprising the nucleic acid molecule of any one of claims 1 to 10.

12. A host transformed or transfected with the vector of claim 11.

13. A method of producing a (poly)peptide encoded by the nucleic acid molecule of any one of claims 1 to 10 comprising culturing the host of claim 12 under suitable conditions and isolating said (poly)peptide from the culture.

14. A (poly)peptide encoded by the nucleic acid molecule of any of claims 1 to 10 or produced by the method of claim 13.

15. An antibody or fragment or derivative thereof comprising at least one (poly)peptide of claim 14.

16. The antibody of claim 15 which is a monoclonal antibody.

17. The antibody of claim 15 which is a bispecific antibody.

18. The antibody of claim 17 wherein the first specificity is for the extracellular domain of the human zeta-chain on the surface of an intact cell and the second specificity is for an optionally different molecule on the surface of a T-lymphocyte, a natural killer cell or a precursor thereof.

19. The antibody of claim 17 wherein the first specificity is for the extracellular domain of the human zeta-chain on the surface of an intact cell and the second specificity is for a different molecule on the surface of a different cell, preferably a cell different from a T-cell, an NK-cell or a precursor thereof, wherein preferably said different molecule is a virus encoded antigen, a tumor associated antigen or a surface antigen either on antigen presenting cells (APCs), most preferably dendritic cells, or on non-APCs.

20. The derivative of claim 15 which is an scFv chain.

21. The antibody of claim 16 which is an IgM.

22. A bispecific receptor comprising a (poly)peptide of claim 14 and a natural receptor, natural ligand or derivatives thereof interacting with a surface molecule on the same or on another cell, wherein preferably said receptors or ligands are CD4, CTLA-4, B7-1, B7-2, LFA-3, ICAM-1, -2, -3 or chemokines like MIP-1α, MIP-1β, RANTES or SDF-1.

23. A pharmaceutical composition comprising the nucleic acid molecule of any of claims 1 to 10, the vector of claim 11, the host of claim 12, the (poly)peptide of claim 14, the antibody or fragment or derivative thereof of any one of claims 15 to 21 and/or the bispecific receptor of claim 22.

24. Use of the antibody of claim 18 for the preparation of a pharmaceutical composition for the treatment or prevention of autoimmune diseases, immune deficiencies, T-cell malignancies, infectious diseases or for the suppression of immune response preferably in order to avoid graft rejection after organ transplantation.

25. Use of the antibody claim 19 for the preparation of a pharmaceutical composition of the treatment or prevention of malignancies, viral infections, or other infectious diseases.

26. Use of the (poly)peptide of claim 14 or the antibody or fragment or derivative thereof of any one of claims 15 to 21 or the bispecific receptor of claim 22 for the preparation of a pharmaceutical composition for the enhancement or suppression of NK-cell dependent immunity or for the treatment of NK-cell derived malignancies.

27. A method for the determination of zeta-chain or eta-chain expression on NK-cells, T-lymphocytes or precursors thereof comprising
    (a) contacting the (poly)peptide of claim 14 or the antibody or fragment or derivative thereof of any one claims 15 to 21 with said NK-cells, T-lymphocytes or precursors thereof; and
    (b) assessing the amount of bound (poly)peptide, antibody or derivative.

28. A kit comprising the nucleic acid molecule of any of claims 1 to 10, the vector of claim 11, the host of claim 12, the (poly)peptide of claim 14, the antibody or fragment or derivative thereof of any one of claims 15 to 21 and/or the bispecific receptor of claim 22.

29. A transgenic animal comprising in its germline at least one copy of the nucleic acid molecule of any of claims 1 to 10 or the vector of claim 11.

Table 1:

murine V heavy chain:

5'-primer	
MVH1	5'- (GC) AGGTGCAGCTCGAGGAGTCAGGACCT-3'
MVH2	5'-GAGGTCCAGCTCGAGCAGTCTGGACCT-3'
MVH3	5'-CAGGTCCAACTCGAGCAGCCTGGGGCT-3'
MVH4	5'-GAGGTTCAGCTCGAGCAGTCTGGGGCA-3'
MVH5	5'-GA (AG) GTGAAGCTCGAGGAGTCTGGAGGA-3'
MVH6	5'-GAGGTGAAGCTTCTCGAGTCTGGAGGT-3'
MVH7	5'-GAAGTGAAGCTCGAGGAGTCTGGGGGA-3'
MVH8	5'-GAGGTTCAGCTCGAGCAGTCTGGAGCT-3'

3'-primer	
MIgG1	5'-TATGCAACTAGTACAACCACAATCCCTGGG-3'
MIgG2a	5'-GAGAGAGGGGTTCTGACTAGTGGGCACTCTGGGCTC-3'
MIgG2b	5'-CTCCTTACTAGTAGGACAGGGGTTGATTGT-3'
MIgG3	5'-GTTACCACTAGTGCATGAAGAACCTGGGGG-3'

murine V kappa chain:

5'-primer	
MUVK1	5'-CCAGTTCCGAGCTCGTTGTGACTCAGGAATCT-3'
MUVK2	5'-CCAGTTCCGAGCTCGTGTTGACGCAGCCGCCC-3'
MUVK3	5'-CCAGTTCCGAGCTCGTGCTCACCCAGTCTCCA-3'
MUVK4	5'-CCAGTTCCGAGCTCCAGATGACCCAGTCTCCA-3'
MUVK5	5'-CCAGATGTGAGCTCGTGATGACCCAGACTCCA-3'
MUVK6	5'-CCAGATGTGAGCTCGTCATGACCCAGTCTCCA-3'
MUVK7	5'-CCAGTTCCGAGCTCGTGATGACACAGTCTCCA-3'

3'-primer	
MUCK	5'-GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA -3'

Fig. 1: Structure of the TCR: schematic representation of early events in T-cell activation

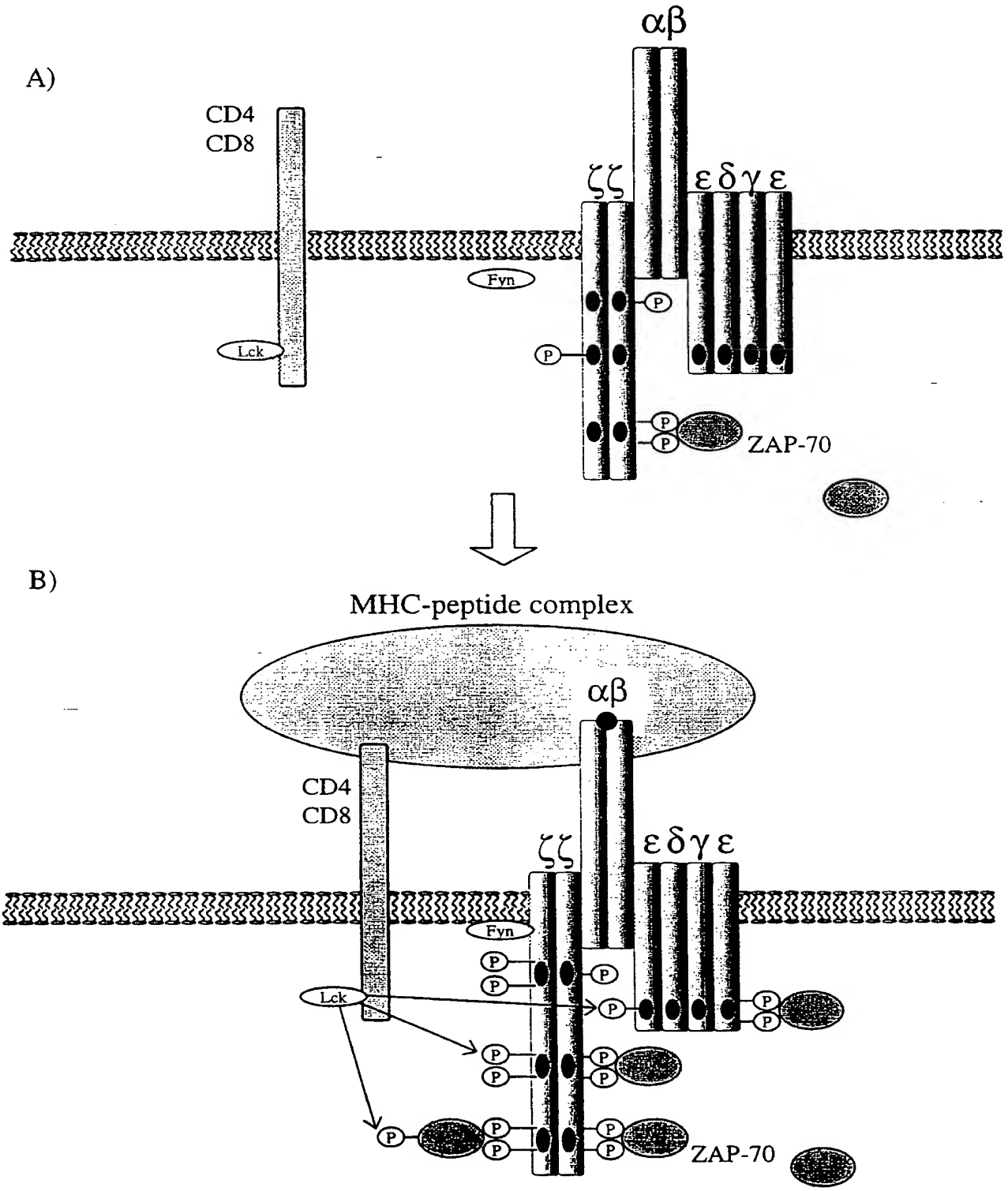


Fig. 2:

ELISA-analysis of zeta-chain specific Fab-antibody-fragments
selected by phage display technique:
Binding ability on soluble zeta-peptide-BSA-conjugate

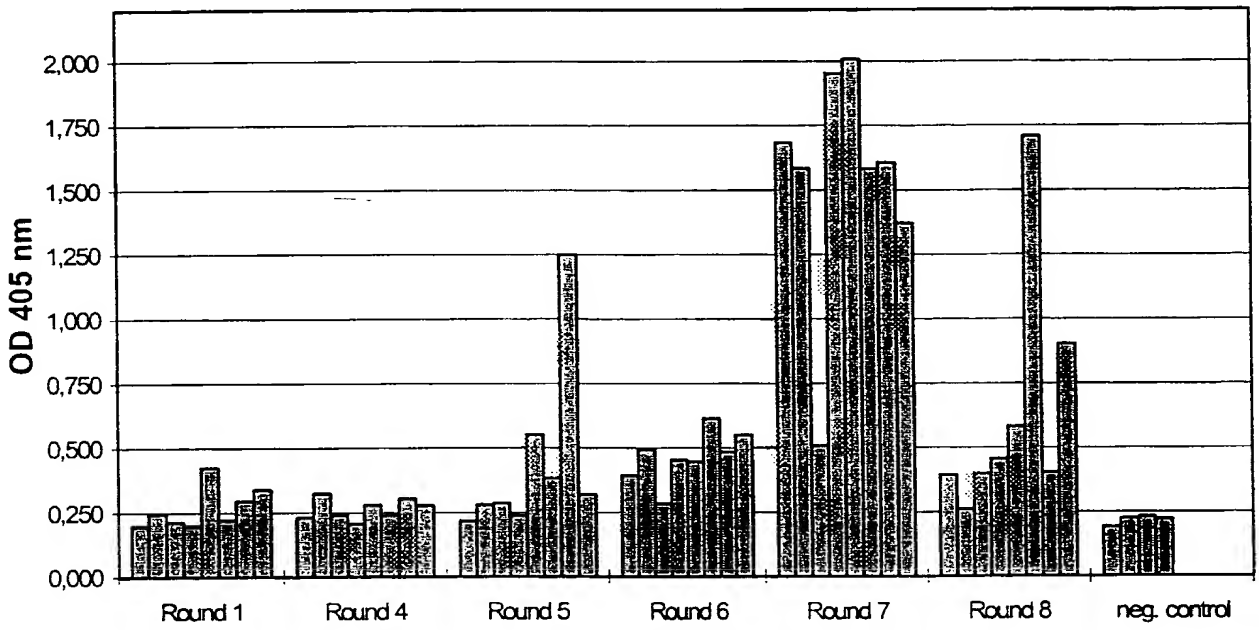


Fig. 3a: **CD8⁺-T-lymphocytes stained with 2-B-5 hybridoma culture supernatant**

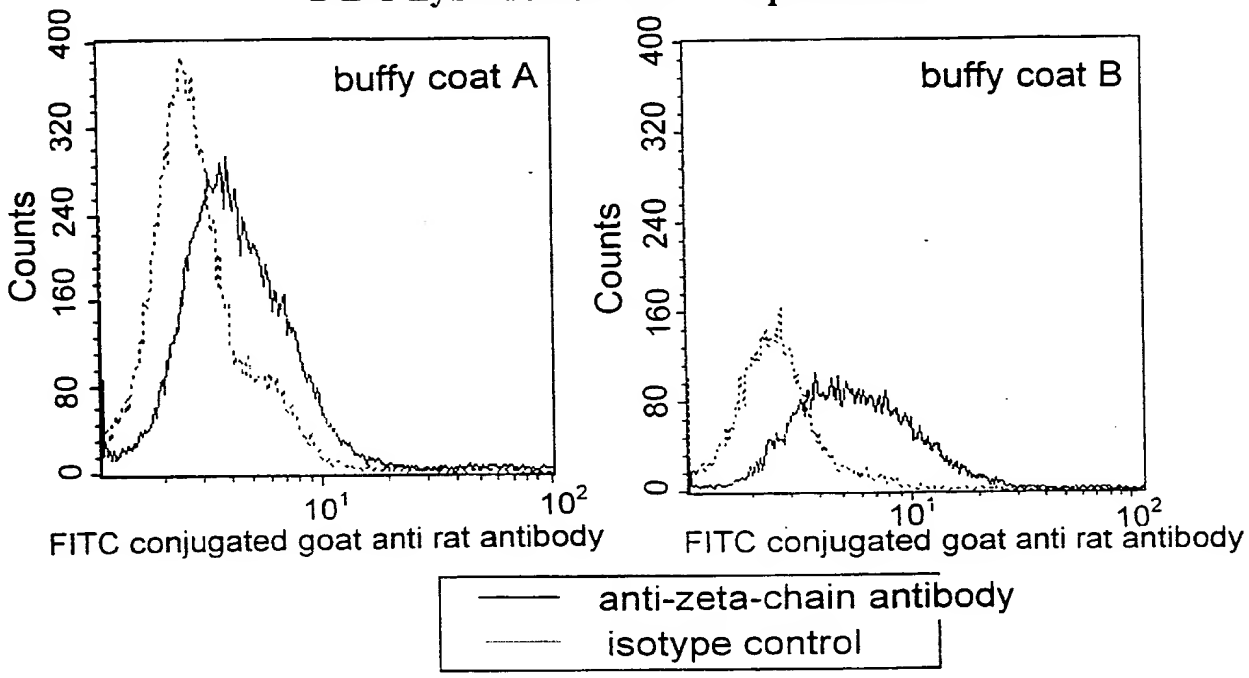


Fig. 3b: **CD56⁺ NK-cells stained with 2-B-5 hybridoma culture supernatant**

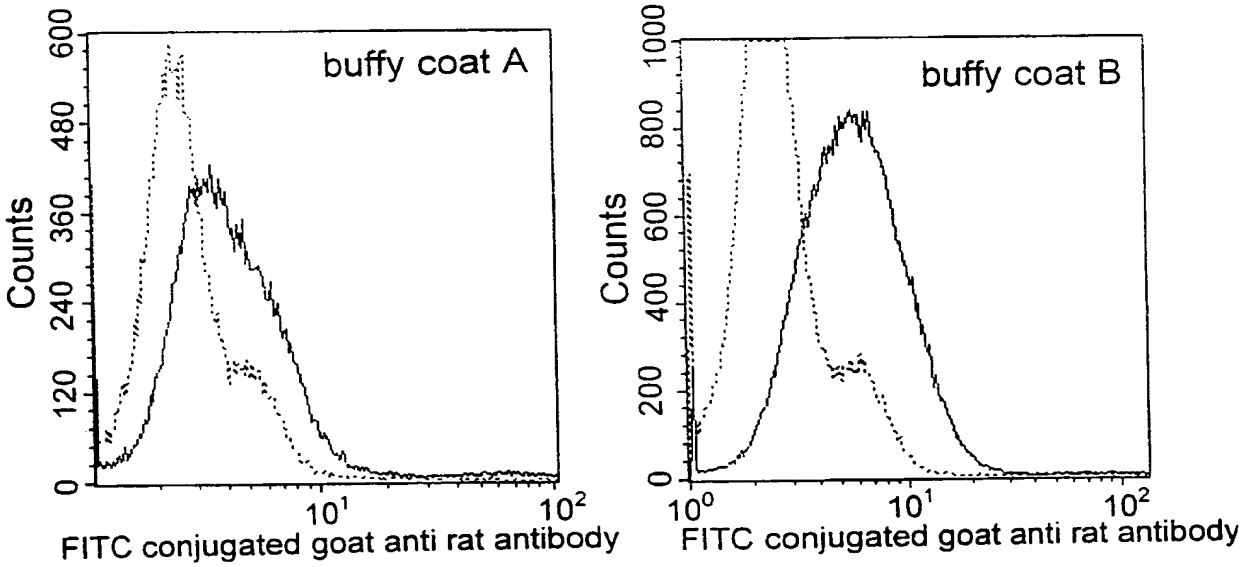


Fig. 4:

ELISA-analysis
Detection of anti-zeta-chain specific binding activity on zeta-chain-isolates derived from CD8$^+$-T-cell lysates

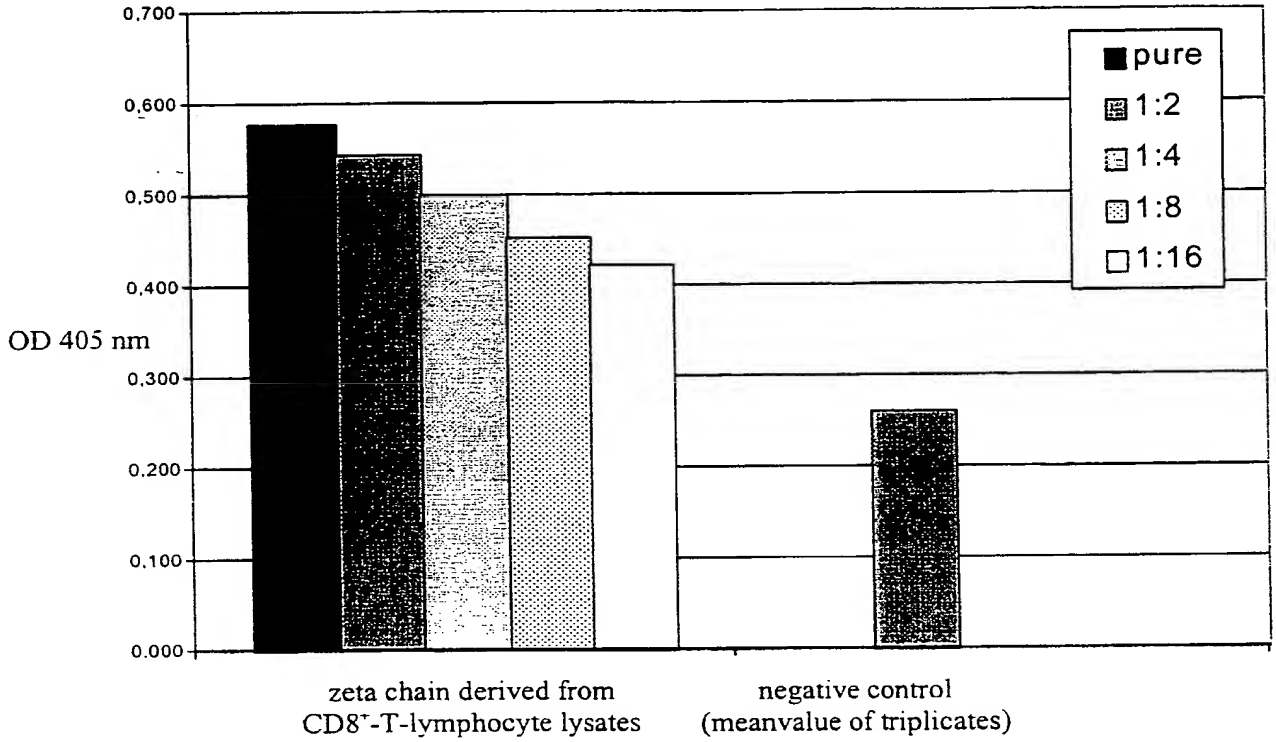


Fig. 5:

ELISA-analysis:
Binding ability of periplasmatically expressed Fab-Fragments with anti-zeta-chain specificity on soluble zeta-KLH conjugate

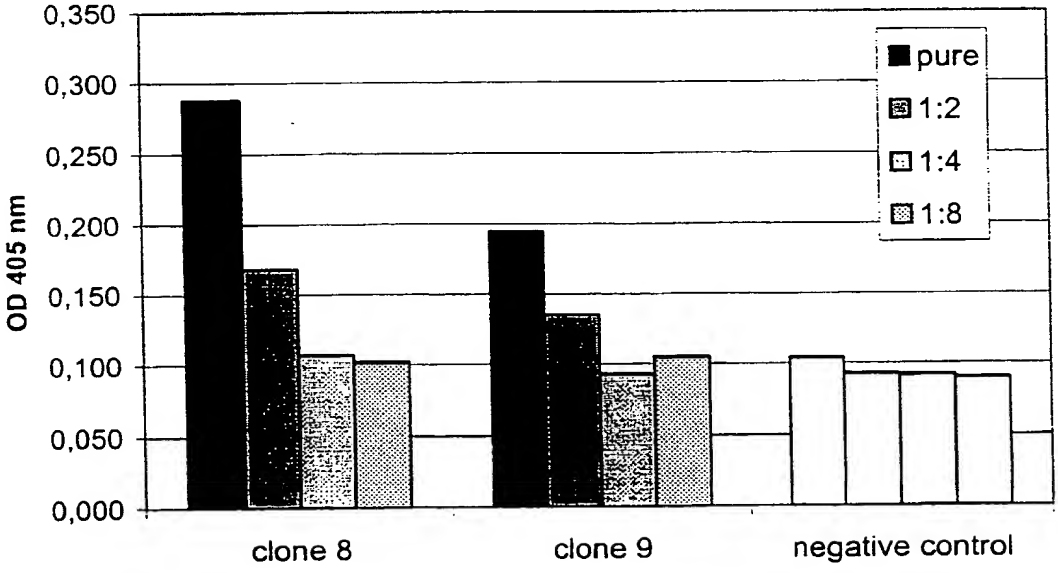


Fig. 6:

```
                9         18        27        36        45        54
5'  CAG GTA CAG CTG CAG CAA TCT GGG GCT GAA CTA GTG AAG CCT GGG TCC TCA GTG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     Q   V   Q   L   Q   Q   S   G   A   E   L   V   K   P   G   S   S   V
```

CDR 1

```
            63        72        81        90        99        108
    AAA ATT TCC TGC AAG GCT TCT GGC TAC ACA TTC ACC AGT TAC GAT ATG CAC TGG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     K   I   S   C   K   A   S   G   Y   T   F   T   S   Y   D   M   H   W

        117       126       135       144       153       162
    ATA AAA CAG CAG CCT GGA AAT GGC CTT GAG TGG ATT GGG TGG ATT TAT CCT GGA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     I   K   Q   Q   P   G   N   G   L   E   W   I   G   W   I   Y   P   G
```

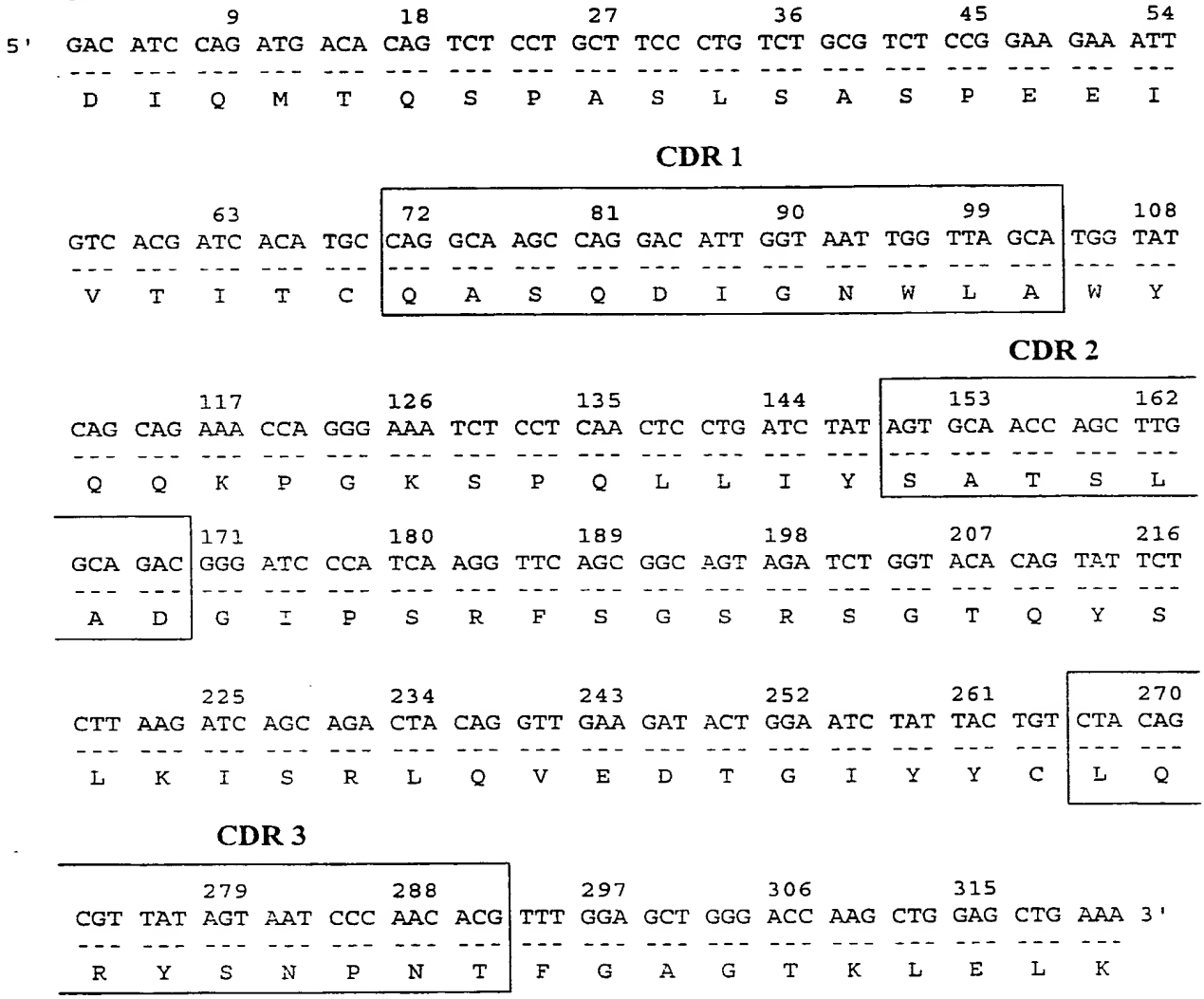
CDR 2

```
            171       180       189       198               207       216
    AAT GGT AAT ACT AAG TAC AAT CAA AAG TTC AAT GGG AAG GCA ACA CTC ACT GCA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     N   G   N   T   K   Y   N   Q   K   F   N   G   K   A   T   L   T   A

            225       234       243       252       261       270
    GAC AAA TCC TCC AGC ACA GCC TAT ATG CAG CTC AGC AGC CTG ACA TCT GAG GAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     D   K   S   S   S   T   A   Y   M   Q   L   S   S   L   T   S   E   D
```

CDR 3

```
            279       288       297       306       315       324
    TCT GCA GTC TAT TTC TGT GCA AGA GAT TGG CAT TAC TAT AGC AGC TAT ATC CGT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     S   A   V   Y   F   C   A   R   D   W   H   Y   Y   S   S   Y   I   R

            333       342       351       360       369
    CCC TTT GCT TAC TGG GGC CAA GGC ACT CTG GTC ACT GTC TCT TCA 3'
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     P   F   A   Y   W   G   Q   G   T   L   V   T   V   S   S
```

Fig. 7:



		9			18			27			36			45			54		
5'	GAC	ATC	CAG	ATG	ACA	CAG	TCT	CCT	GCT	TCC	CTG	TCT	GCG	TCT	CCG	GAA	GAA	ATT	
	D	I	Q	M	T	Q	S	P	A	S	L	S	A	S	P	E	E	I	
			63			72			81			90			99			108	
	GTC	ACG	ATC	ACA	TGC	CAG	GCA	AGC	CAG	GAC	ATT	GGT	AAT	TGG	TTA	GCA	TGG	TAT	
	V	T	I	T	C	Q	A	S	Q	D	I	G	N	W	L	A	W	Y	
			117			126			135			144			153			162	
	CAG	CAG	AAA	CCA	GGG	AAA	TCT	CCT	CAA	CTC	CTG	ATC	TAT	AGT	GCA	ACC	AGC	TTG	
	Q	Q	K	P	G	K	S	P	Q	L	L	I	Y	S	A	T	S	L	
	GCA	GAC	171			180			189			198			207			216	
			GGG	ATC	CCA	TCA	AGG	TTC	AGC	GGC	AGT	AGA	TCT	GGT	ACA	CAG	TAT	TCT	
	A	D	G	I	P	S	R	F	S	G	S	R	S	G	T	Q	Y	S	
			225			234			243			252			261			270	
	CTT	AAG	ATC	AGC	AGA	CTA	CAG	GTT	GAA	GAT	ACT	GGA	ATC	TAT	TAC	TGT	CTA	CAG	
	L	K	I	S	R	L	Q	V	E	D	T	G	I	Y	Y	C	L	Q	
			279			288			297			306			315				
	CGT	TAT	AGT	AAT	CCC	AAC	ACG	TTT	GGA	GCT	GGG	ACC	AAG	CTG	GAG	CTG	AAA	3'	
	R	Y	S	N	P	N	T	F	G	A	G	T	K	L	E	L	K		

Fig. 8:

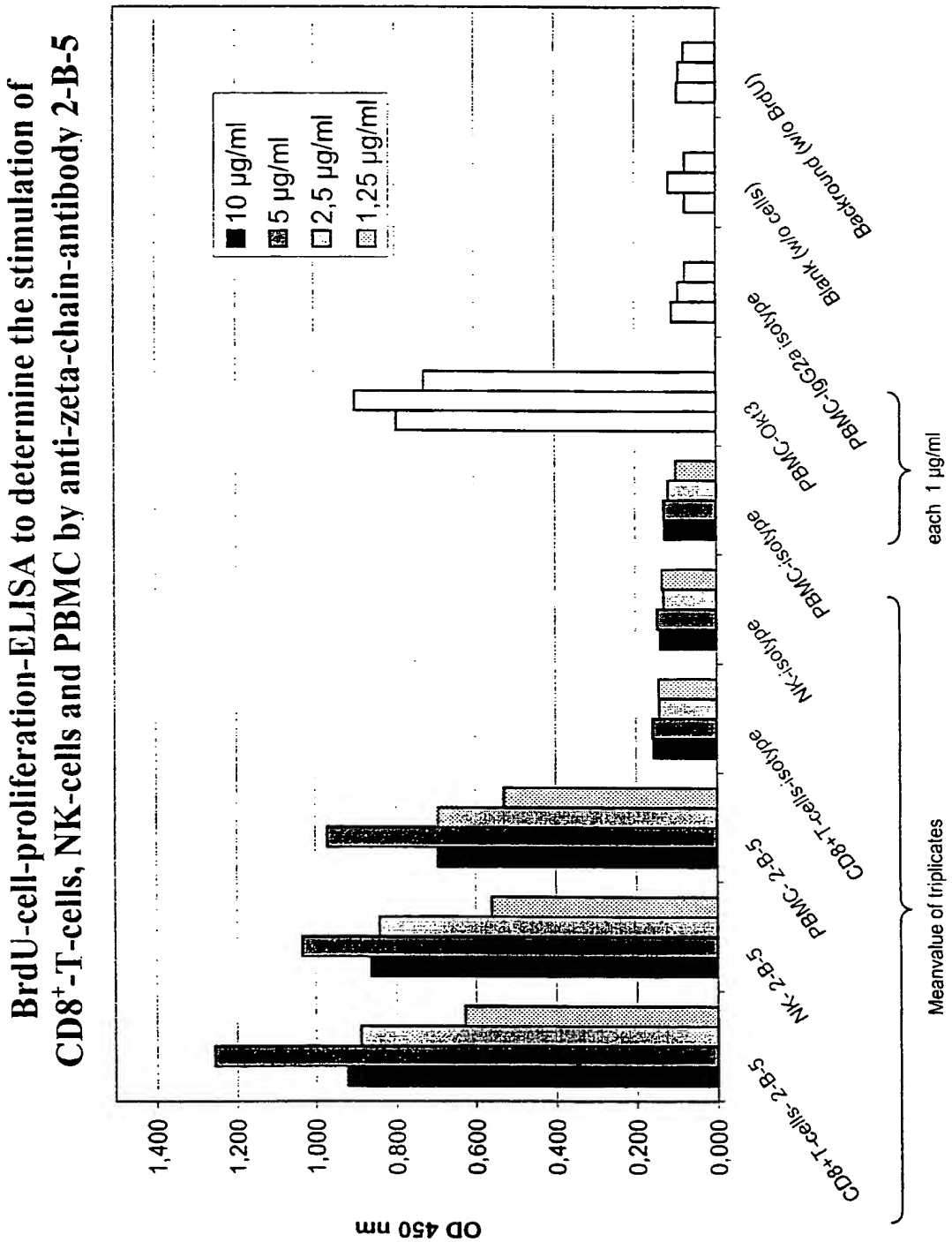

BrdU-cell-proliferation-ELISA to determine the stimulation of CD8+-T-cells, NK-cells and PBMC by anti-zeta-chain-antibody 2-B-5
10 µg/ml
5 µg/ml
2,5 µg/ml
1,25 µg/ml
OD 450 nm
1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
CD8+T-cells-2-B-5
NK-2-B-5
PBMC-2-B-5
CD8+T-cells-isotype
NK-isotype
PBMC-isotype
PBMC-OKt3
PBMC-IgG2a isotype
Blank (w/o cells)
Background (w/o BrdU)
Meanvalue of triplicates
each 1 µg/ml

Fig. 9a:

PBMC stained with the anti-zeta 2B5 antibody
at different temperatures and incubation times

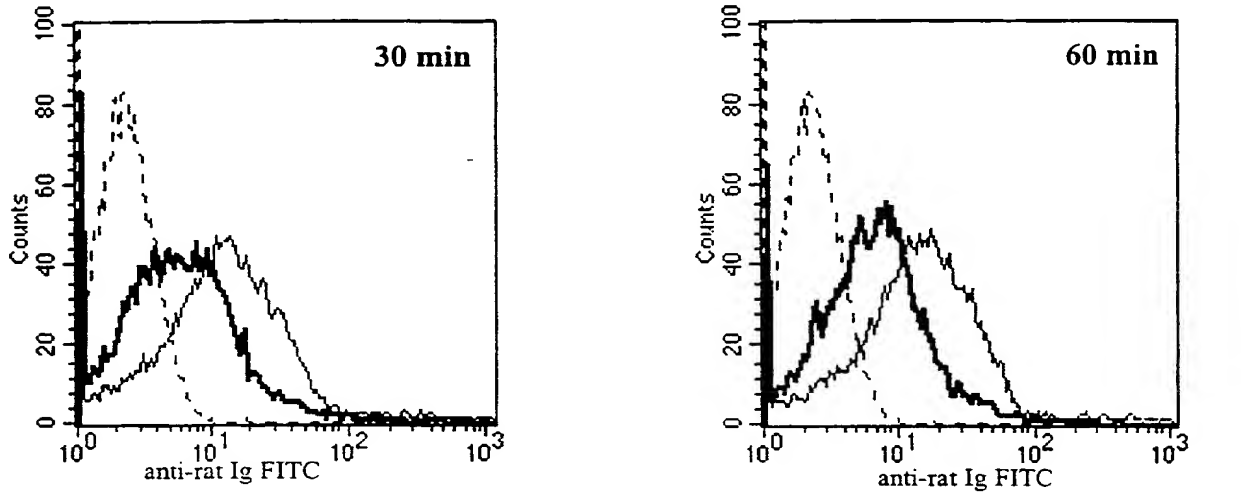


Fig. 9b: PBMC stained with the anti-CD3 antibody
at different temperatures and incubation times

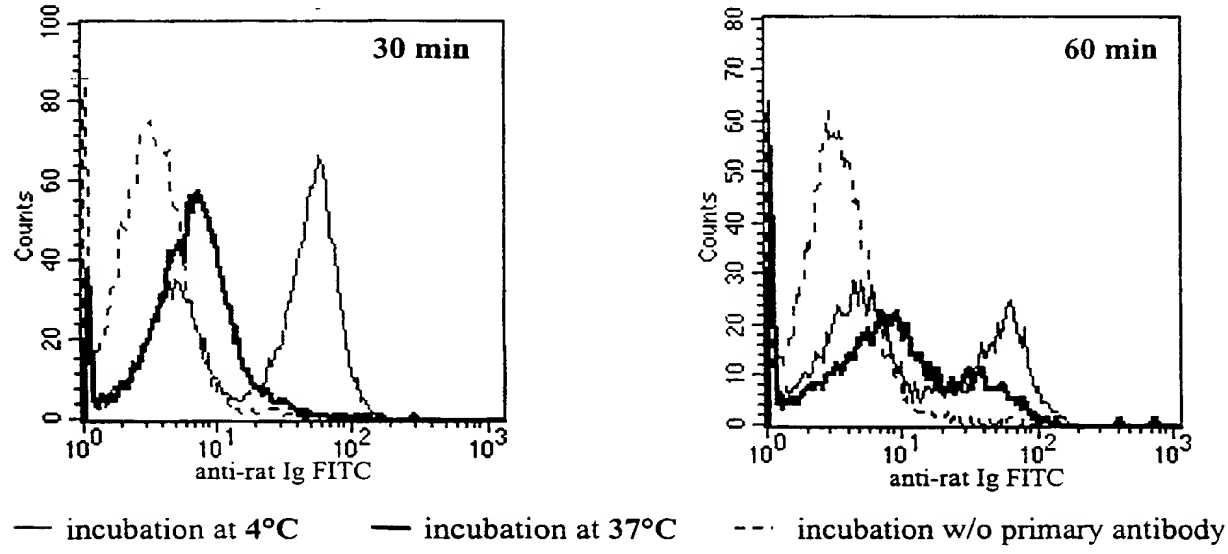
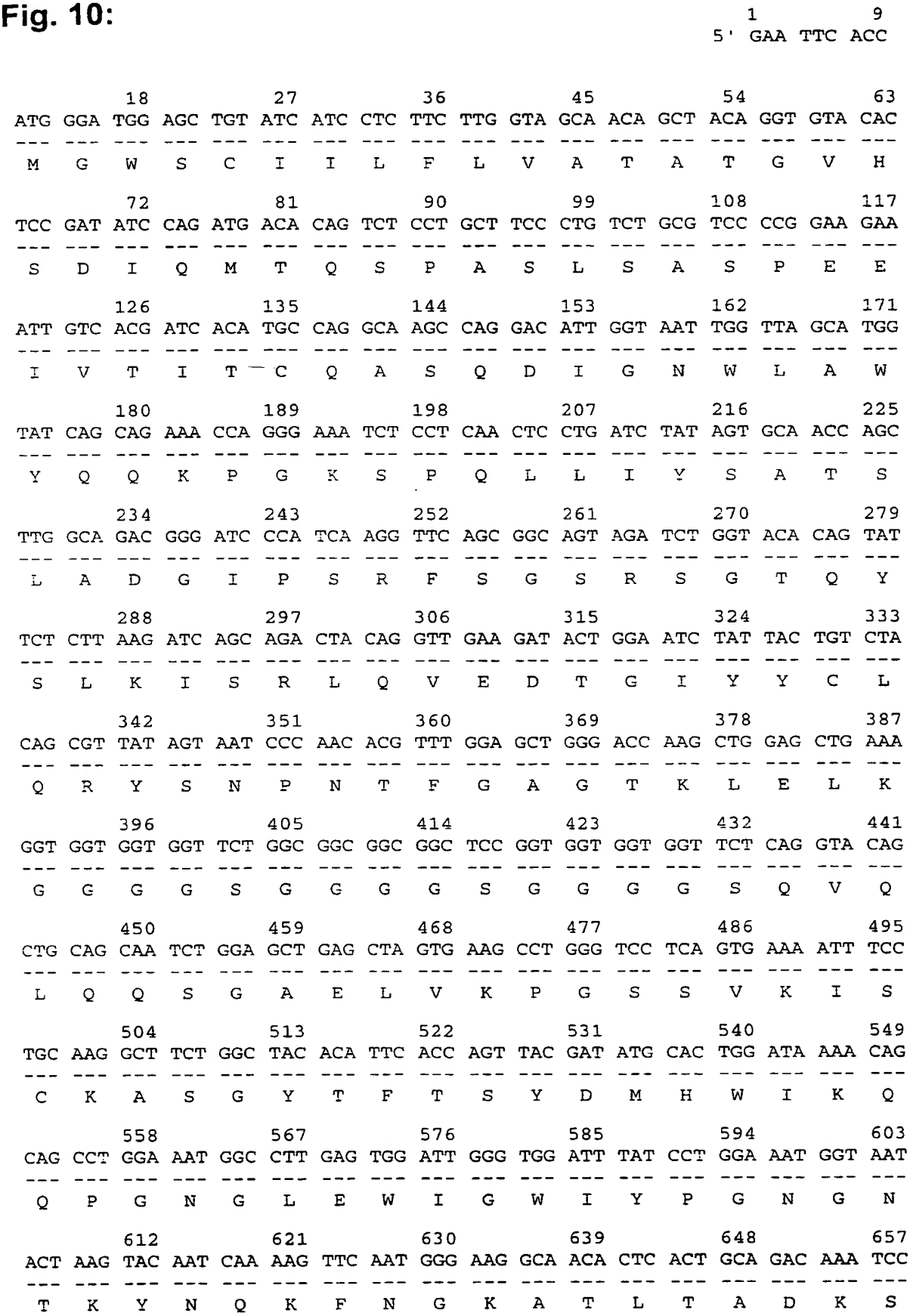


Fig. 10: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TCC GAT ATC CAG ATG ACA CAG TCT CCT GCT TCC CTG TCT GCG TCC CCG GAA GAA
S D I Q M T Q S P A S L S A S P E E
126 135 144 153 162 171
ATT GTC ACG ATC ACA TGC CAG GCA AGC CAG GAC ATT GGT AAT TGG TTA GCA TGG
I V T I T C Q A S Q D I G N W L A W
180 189 198 207 216 225
TAT CAG CAG AAA CCA GGG AAA TCT CCT CAA CTC CTG ATC TAT AGT GCA ACC AGC
Y Q Q K P G K S P Q L L I Y S A T S
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TTG GCA GAC GGG ATC CCA TCA AGG TTC AGC GGC AGT AGA TCT GGT ACA CAG TAT
L A D G I P S R F S G S R S G T Q Y
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G G G G S G G G G S G G G G S Q V Q
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612 621 630 639 648 657
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T K Y N Q K F N G K A T L T A D K S

Fig. 10 (cont.):

```
          666        675        684        693        702        711
TCC AGC ACA GCC TAT ATG CAG CTC AGC AGC CTG ACA TCT GAG GAC TCT GCA GTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   S   T   A   Y   M   Q   L   S   S   L   T   S   E   D   S   A   V

          720        729        738        747        756        765
TAT TTC TGT GCA AGA GAT TGG CAT TAC TAT AGC AGC TAT ATC CGT CCC TTT GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Y   F   C   A   R   D   W   H   Y   Y   S   S   Y   I   R   P   F   A

          774        783        792        801        810        819
TAC TGG GGC CAA GGC ACT CTG GTC ACT GTC TCT TCC GGA GGT GGT GGT TCT GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Y   W   G   Q   G   T   L   V   T   V   S   S   G   G   G   G   S   E

          828        837        846        855        864        873
GTG CAG CTG CTC GAG CAG TCT GGA GCT GAG CTG GCG AGG CCT GGG GCT TCA GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   Q   L   L   E   Q   S   G   A   E   L   A   R   P   G   A   S   V

          882        891        900        909        918        927
AAG CTG TCC TGC AAG GCT TCT GGC TAC ACC TTC ACA AAC TAT GGT TTA AGC TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   L   S   C   K   A   S   G   Y   T   F   T   N   Y   G   L   S   W

          936        945        954        963        972        981
GTG AAG CAG AGG CCT GGA CAG GTC CTT GAG TGG ATT GGA GAG GTT TAT CCT AGA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   K   Q   R   P   G   Q   V   L   E   W   I   G   E   V   Y   P   R

          990        999       1008       1017       1026       1035
ATT GGT AAT GCT TAC TAC AAT GAG AAG TTC AAG GGC AAG GCC ACA CTG ACT GCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   G   N   A   Y   Y   N   E   K   F   K   G   K   A   T   L   T   A

         1044       1053       1062       1071       1080       1089
GAC AAA TCC TCC AGC ACA GCG TCC ATG GAG CTC CGC AGC CTG ACC TCT GAG GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   K   S   S   S   T   A   S   M   E   L   R   S   L   T   S   E   D

         1098       1107       1116       1125       1134       1143
TCT GCG GTC TAT TTC TGT GCA AGA CGG GGA TCC TAC GAT ACT AAC TAC GAC TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   A   V   Y   F   C   A   R   R   G   S   Y   D   T   N   Y   D   W

         1152       1161       1170       1179       1188       1197
TAC TTC GAT GTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GGT GGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Y   F   D   V   W   G   Q   G   T   T   V   T   V   S   S   G   G   G

         1206       1215       1224       1233       1242       1251
GGT TCT GGC GGC GGC GGC TCC GGT GGT GGT GGT TCT GAG CTC GTG ATG ACC CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   S   G   G   G   G   S   G   G   G   G   S   E   L   V   M   T   Q

         1260       1269       1278       1287       1296       1305
ACT CCA CTC TCC CTG CCT GTC AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   P   L   S   L   P   V   S   L   G   D   Q   A   S   I   S   C   R
```

Fig. 10 (cont.):

```
      1314        1323        1332        1341        1350        1359
TCT AGT CAG AGC CTT GTA CAC AGT AAT GGA AAC ACC TAT TTA CAT TGG TAC CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   S   Q   S   L   V   H   S   N   G   N   T   Y   L   H   W   Y   L

      1368        1377        1386        1395        1404        1413
CAG AAG CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   K   P   G   Q   S   P   K   L   L   I   Y   K   V   S   N   R   F

      1422        1431        1440        1449        1458        1467
TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   G   V   P   D   R   F   S   G   S   G   S   G   T   D   F   T   L

      1476        1485        1494        1503        1512        1521
AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   I   S   R   V   E   A   E   D   L   G   V   Y   F   C   S   Q   S

      1530        1539        1548        1557        1566        1575
ACA CAT GTT CCG TAC ACG TTC GGA GGG GGG ACC AAG CTT GAG ATC AAA CGT ACG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   H   V   P   Y   T   F   G   G   G   T   K   L   E   I   K   R   T

      1584        1593        1602        1611        1620        1629
ACT AGC CAT CAC CAT CAC CAT CAC ACT AGC TAA TTA ATT TAA GCG GCC GCT CTA
--- --- --- --- --- --- --- --- --- --- ---
 T   S   H   H   H   H   H   H   T   S   *

GAG TCG AC 3'
```

Fig. 11:

Cytotoxic activity of PBMC and CD8$^+$-T-cells redirected against EpCAM-positive Kato cells by the anti-zeta-chain/anti-EpCAM antibody

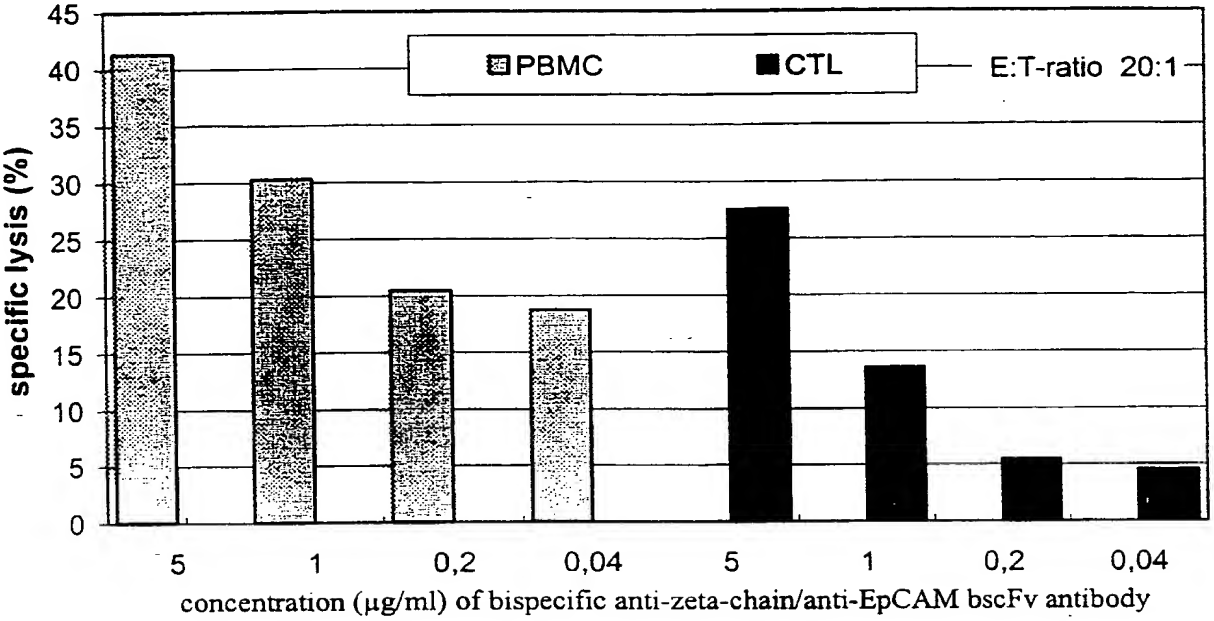
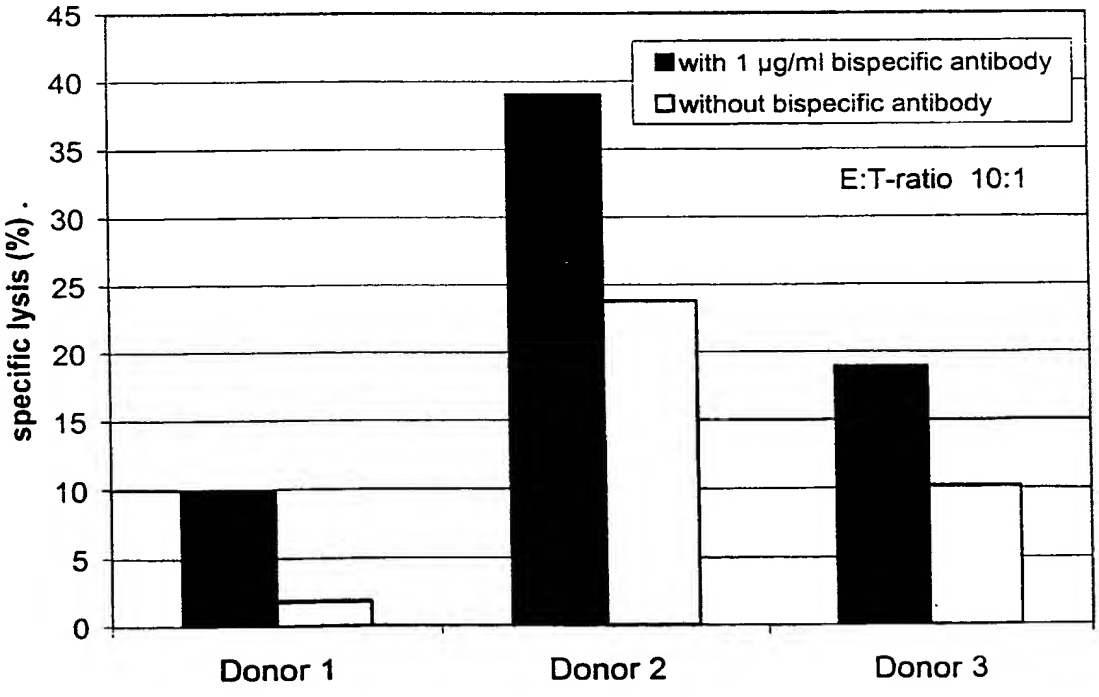


Fig. 12:

Cytotoxic activity of NK-cells redirected against EpCAM-positive Kato cells by the bispecific anti-zeta-chain/anti-EpCAM antibody



SEQUENCE LISTING

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Gly

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Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

gat atg cac tgg ata aaa cag cag cct gga aat ggc ctt gag tgg att   144
Asp Met His Trp Ile Lys Gln Gln Pro Gly Asn Gly Leu Glu Trp Ile
        35                  40                  45

ggg tgg att tat cct gga aat ggt aat act aag tac aat caa aag ttc   192
Gly Trp Ile Tyr Pro Gly Asn Gly Asn Thr Lys Tyr Asn Gln Lys Phe
    50                  55                  60

aat ggg aag gca aca ctc act gca gac aaa tcc tcc agc aca gcc tat   240
Asn Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

atg cag ctc agc agc ctg aca tct gag gac tct gca gtc tat ttc tgt   288
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                  90                  95

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Ala Arg Asp Trp His Tyr Tyr Ser Ser Tyr Ile Arg Pro Phe Ala Tyr
            100                 105                 110

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            20                  25                  30

Asp Met His Trp Ile Lys Gln Gln Pro Gly Asn Gly Leu Glu Trp Ile
        35                  40                  45

Gly Trp Ile Tyr Pro Gly Asn Gly Asn Thr Lys Tyr Asn Gln Lys Phe
    50                  55                  60

Asn Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                  90                  95

Ala Arg Asp Trp His Tyr Tyr Ser Ser Tyr Ile Arg Pro Phe Ala Tyr
            100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120
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gaa att gtc acg atc aca tgc cag gca agc cag gac att ggt aat tgg   96
Glu Ile Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Gly Asn Trp
            20                  25                  30

tta gca tgg tat cag cag aaa cca ggg aaa tct cct caa ctc ctg atc   144
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ser Pro Gln Leu Leu Ile
         35                  40                  45

tat agt gca acc agc ttg gca gac ggg atc cca tca agg ttc agc ggc   192
Tyr Ser Ala Thr Ser Leu Ala Asp Gly Ile Pro Ser Arg Phe Ser Gly
     50                  55                  60

agt aga tct ggt aca cag tat tct ctt aag atc agc aga cta cag gtt   240
Ser Arg Ser Gly Thr Gln Tyr Ser Leu Lys Ile Ser Arg Leu Gln Val
 65                  70                  75                  80

gaa gat act gga atc tat tac tgt cta cag cgt tat agt aat ccc aac   288
Glu Asp Thr Gly Ile Tyr Tyr Cys Leu Gln Arg Tyr Ser Asn Pro Asn
                85                  90                  95

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            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ser Pro Gln Leu Leu Ile
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Tyr Ser Ala Thr Ser Leu Ala Asp Gly Ile Pro Ser Arg Phe Ser Gly
     50                  55                  60

Ser Arg Ser Gly Thr Gln Tyr Ser Leu Lys Ile Ser Arg Leu Gln Val
 65                  70                  75                  80

Glu Asp Thr Gly Ile Tyr Tyr Cys Leu Gln Arg Tyr Ser Asn Pro Asn
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Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys
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35 40 45

Gly Asn Trp Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ser Pro Gln

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     50                  55                  60

Leu Leu Ile Tyr Ser Ala Thr Ser Leu Ala Asp Gly Ile Pro Ser Arg
 65                  70                  75                  80

Phe Ser Gly Ser Arg Ser Gly Thr Gln Tyr Ser Leu Lys Ile Ser Arg
                 85                  90                  95

Leu Gln Val Glu Asp Thr Gly Ile Tyr Tyr Cys Leu Gln Arg Tyr Ser
            100                 105                 110

Asn Pro Asn Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Gly Gly
        115                 120                 125

Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln Val Gln
    130                 135                 140

Leu Gln Gln Ser Gly Ala Glu Leu Val Lys Pro Gly Ser Ser Val Lys
145                 150                 155                 160

Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asp Met His
                165                 170                 175

Trp Ile Lys Gln Gln Pro Gly Asn Gly Leu Glu Trp Ile Gly Trp Ile
            180                 185                 190

Tyr Pro Gly Asn Gly Asn Thr Lys Tyr Asn Gln Lys Phe Asn Gly Lys
        195                 200                 205

Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu
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Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Ala Arg Asp
225                 230                 235                 240

Trp His Tyr Tyr Ser Ser Tyr Ile Arg Pro Phe Ala Tyr Trp Gly Gln
                245                 250                 255

Gly Thr Leu Val Thr Val Ser Ser Gly Gly Gly Gly Ser Glu Val Gln
            260                 265                 270

Leu Leu Glu Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala Ser Val
        275                 280                 285

Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr Gly Leu
    290                 295                 300

Ser Trp Val Lys Gln Arg Pro Gly Gln Val Leu Glu Trp Ile Gly Glu
305                 310                 315                 320

Val Tyr Pro Arg Ile Gly Asn Ala Tyr Tyr Asn Glu Lys Phe Lys Gly
                325                 330                 335

Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Ser Met Glu
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Gly Gly Ser Gly Gly Gly Gly Ser Glu Leu Val Met Thr Gln Thr Pro
405 410 415

Leu Ser Leu Pro Val Ser Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg
420 425 430

Ser Ser Gln Ser Leu Val His Ser Asn Gly Asn Thr Tyr Leu His Trp
435 440 445

Tyr Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val
450 455 460

Ser Asn Arg Phe Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser
465 470 475 480

Gly Thr Asp Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Leu
485 490 495

Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro Tyr Thr Phe Gly
500 505 510

Gly Gly Thr Lys Leu Glu Ile Lys Arg Thr Thr Ser His His His His
515 520 525

His His Thr Ser
530

INTERNATIONAL SEARCH REPORT

International Application No
PC., EP 99/04838

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/13 C12N15/70 C12N15/85 C12N1/21 C12N5/10
C07K16/28 C07K16/46 A61K31/70 A61K39/395 G01N33/577
G01N33/68 C12Q1/68 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M. MACK ET AL.: "Biologic properties of a bispecific single-chain antibody directed against 17-1A (EpCAM) and CD3: tumor cell-dependent T cell stimulation and cytotoxic activity." THE JOURNAL OF IMMUNOLOGY, vol. 158, no. 8, 15 April 1997 (1997-04-15), pages 3965-3970, XP002100040 Baltimore, MD, USA cited in the application the whole document --- -/--	1-29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 November 1999	12/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	A. TRAUNECKER ET AL.: "Bispecific single chain molecules (Janusins) target cytotoxic lymphocytes on HIV infected cells." THE EMBO JOURNAL, vol. 10, no. 12, December 1991 (1991-12), pages 3655-3659, XP000232579 Oxford, GB abstract figure 1 ---	1-29
A	W. HELFRICH ET AL.: "Construction and characterization of a bispecific diabody for retargeting T cells to human carcinomas." INTERNATIONAL JOURNAL OF CANCER, vol. 76, no. 2, 13 April 1998 (1998-04-13), pages 232-239, XP002121156 Copenhagen, Denmark abstract material and methods figure 4 ---	1-29
A	C. RENNER ET AL.: "T cells from patients with Hodgkin's disease have a defective T-cell receptor zeta chain expression that is reversible by T-cell stimulation with CD3 and CD28." BLOOD, vol. 88, no. 1, 1 July 1996 (1996-07-01), pages 236-241, XP002121157 New York, NY, USA abstract page 237, left-hand column, line 1 - line 14 ---	1-29
A	J. SMITH ET AL.: "Nonmitogenic anti-CD3 monoclonal antibodies deliver a partial T cell recetor signal and induce clonal anergy." THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 185, no. 8, 21 April 1997 (1997-04-21), pages 1413-1422, XP002121158 New York, NY, USA abstract page 1414, right-hand column, line 12 - line 45 ---	1-29
A	WO 90 15822 A (DANA-FARBER CANCER INSTITUTE) 27 December 1990 (1990-12-27) the whole document -----	1-29

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PC./EP 99/04838

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9015822 A	27-12-1990	NONE	